Remarks:

Claims:

By the present amendment, claims 61 and 64 have been amended to more particularly and distinctly define the invention. New claims 69-71 have been added to claim particular embodiments of the invention. The number of total claims and independent claims remains less than the number of claims previously paid for.

Support for the new claims is either apparent, or is as indicated below. Support for new claims 69 and 70 can be found, for example, in Example 8 at pages 63-64. SEQ ID NO: 9 and SEQ ID NO: 10 include the amino acid sequence recited in claims 69 and 70, respectively. It is noted that each of SEQ ID NO: 9 and SEQ ID NO: 10 include a terminal cysteine residue that is absent in SEQ ID NO:2, and that facilitates coupling to the carrier protein. No new matter is added.

Reconsideration of the rejections is respectfully requested.

Claim Rejections - 35 U.S.C. §112, First Paragraph -Written Description

Claims 61-64 and 66-68 stand rejected under 35 U.S.C. §112, first paragraph based on an assertion that the claims contained subject matter that was not described in the specification in such a way as to reasonably convey to one of skill in the art that the inventor, at the time, the application was filed, had possession of the claimed invention. In particular, the Office asserted that the specification does not teach a fragment sequence that is able to induce an antibody, and that this induced antibody is able to recognize the polypeptide SEQ ID NO:2. The Office further alleges that the specification fails to teach the structure or relevant identifying characteristics of fragments of SEQ ID NO:2, sufficient to allow one of skill in the art to determine that the inventor had possession of the invention as claimed.

Applicant respectfully disagrees. Applicant submits that the Notice, entitled, "*Guidelines for Examination of Patent Applications under the 35 U.S.C. 112, ¶1. Written Description" Requirement* at p. 1104, vol 66, no. 4 (January 5, 2001) addresses the written description provision as follows (emphasis added):

> An applicant shows possession of the claimed invention with all its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed

> invention. Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing characteristics sufficient to show that the applicant was in possession of the claimed invention.

Applicant notes that the specification discloses an immunogenic fragment of a BASB027 polypeptide, that is a contiguous portion of the BASB027 polypeptide which has the same or substantially the same immunogenic activity as the polypeptide comprising the amino acid sequence of SEQ ID NO:2 at, for example, page 5, lines 17-21. In addition, the specification further describes preferred fragments including an isolated polypeptide comprising an amino acid sequence having at least 15 or 20 contiguous amino acids of SEQ ID NO:2 at, for example, page 6, lines 18-22. Applicant submits that these recitations of the immunogenic fragments, coupled with the disclosed amino acid sequence of SEQ ID NO:2 establish possession of the invention by showing that the invention was "ready for patenting" by the disclosure of structural chemical formulas that show the invention was complete. Moreover, it is noted that the amendments to claims 61 and 64 more closely comport with the recitations in the specification, *supra*.

Accordingly, reconsideration of the Written Description Requirement rejection under 35 U.S.C. 112, ¶1 is respectfully requested.

Claim Rejections - 35 U.S.C. §112, First Paragraph - Enablement

Claims 61-64 and 66-68 stand rejected under 35 U.S.C. §112, first paragraph based on an assertion that the specification, while being enabling for a polypeptide comprising the sequence of the amino acid SEQ ID NO: 2 and a fusion protein comprising the amino acid sequence SEQ ID NO:2, does not reasonably provide enablement for an isolated polypeptide that comprises a fragment of at least 15 or 20 amino acids, fusion protein or vaccine composition comprising said fragments.

The rejection includes a general discussion of the unpredictability of protein chemistry, and on the consequences of a single change in an amino acid residue on the biological activity of a protein. The specification, according to the Office, has not provided any guidance as to how an artisan would have met the recognized difficulties associated with the unpredictability of protein

chemistry. The rejection concludes by asserting that the skilled artisan would be forced into undue experimentation to practice the invention as claimed.

Applicant respectfully disagrees. Whether the scope of enablement is sufficient is often decided in light of the following factors: (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). These factors are illustrative, not mandatory. Amgen, Inc. v. Chugai Pharm. Co., Ltd., 927 F.2d 1200, 1213, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991). A review of these factors as applied to the present claims, supports Applicant's assertion that the claims are enabled, as outlined in subsections (A) through (G) below.

(A) *Quantity Of Experimentation*

In Reece (Reece et al., 151 J. IMMUNOL. 6175 (1993), attached as Exhibit A),[1] in excess of one thousand (1,304) overlapping 12 residue peptide fragments were synthesized by the multipin method to map T-cell epitopes of tetanus toxin. Pools of 20 peptides each were used to simplify the mapping assays. Thus, it was practical to synthesize a large number of peptides, and the initial screen needed only to assay sixty to seventy pools. Pools that generated strong responses were deconvoluted by assaying the members of the pool. That such experimentation using a multipin method to screen for antigens is ordinary in this art is illustrated in CURRENT PROTOCOLS IN IMMUNOLOGY 9.7.1 (1997) (attached as Exhibit B) and Reece et al., 172 J. IMMUNOL. 241 (1994) (attached as Exhibit C). That such sequence-scanning techniques are ordinary in the art with respect to antibody-mediated antigenicity (as opposed to cellular immunity as in Reece) is illustrated in Geysen et al., 81 PROC. NATL. ACAD. SCI. USA 3998 (1984) (attached as Exhibit D).

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1 The literature cited in this response provides evidence of the state of the art – and is not submitted under 37 CFR §1.56.

Note that in Geysen, antisera to the whole antigen polypeptide was tested for specificity with an extensive scan of specific peptide sequences. This approach is quite useful to the present invention, where the full-length recombinant BASB027 polypeptide that Applicant has isolated can readily be used within the state of the art to produce polyclonal antibodies. These polyclonal antibodies can then be used to screen for promising smaller polypeptide antigens.

(B) *Amount Of Direction Or Guidance Presented*

Guidance can be found in the specification at, for example, page 6, lines 7-16:

> Preferred fragments include, for example, truncation polypeptides having a portion of an amino acid sequence of SEQ ID NO:2 or 4 or of variants thereof, such as a continuous series of residues that includes an amino- and/or carboxyl-terminal amino acid sequence. Degradation forms of the polypeptides of the invention produced by or in a host cell, are also preferred. Further preferred are fragments characterized by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions.

That the sequence-based inferences described here are ordinary in the art, and of known value in selecting positive candidates is illustrated by CURRENT PROTOCOLS IN IMMUNOLOGY 9.3.1 (1991) (attached as Exhibit E).

(C) *Presence Or Absence Of Working Examples*

The specification illustrates the isolation of a full length recombinant BASB027 protein (Example 5), and that the protein is useful in detecting *Moraxella catarrhalis* in human convalescent sera (Example 7). Moreover the specification discloses that two immunogenic fragment of SEQ ID NO:2, one including amino acids 790-804 and one including amino acid 676-691, could be used to raise antibodies that successfully detected SEQ ID NO:2 (Example 8).

(D) *Nature Of The Invention; Predictability Or Unpredictability Of The Art*

The art is no more unpredictable than the chemical arts in general. Thus, the reasonable scope of the claims should be comparable to that which can be achieved with other

structure-focused claims in the chemical arts. Moreover, the ease with which the polypeptides are screened, and the availability of robotic automation tools at the time the application was filed, counterbalance this element of the analysis.

That an unpredictable art nonetheless allows for reasonable inferences of claim scope is illustrated by the following text from the case law:

> Appellants have apparently not disclosed *every* catalyst which will work; they have apparently not disclosed *every* catalyst which will not work. The question, then, is whether in an unpredictable art, section 112 requires disclosure of a test with *every* species covered by a claim. To require such a complete disclosure would apparently necessitate a patent application or applications with "thousands" of examples or the disclosure of "thousands" of catalysts along with information as to whether each exhibits catalytic behavior resulting in the production of hydroperoxides. More importantly, such a requirement would force an inventor seeking adequate patent protection to carry out a prohibitive number of actual experiments. This would tend to discourage inventors from filing patent applications in an unpredictable area since the patent claims would have to be limited to those embodiments which are expressly disclosed. A potential infringer could readily avoid "literal" infringement of such claims by merely finding another analogous catalyst complex which could be used in "forming hydroperoxides."

Application of Angstad, 537 F.2d 498, 502-3, 190 USPQ 214, 218 (CCPA1976) (emphasis in the original).

(E) *State Of The Prior Art*

The highly advanced state of this art is illustrated by the above cited 1984 article by Geysen. The other articles discussed above clearly show that sequence scanning for antigenicity is a highly developed art.

(F) *Relative Skill Of Those In The Art*

In Enzo Biochem, Inc. v. Calgene, Inc., 188 F.3d 1362, 52 USPQ2d 1129 (Fed. Cir. 1999), the Federal Circuit approved a trial court determination in a comparable art that a person of ordinary skill would be a junior faculty member with one or two years of relevant experience

or a postdoctoral student with several years of experience. Applicants respectfully submit that this level of skill is an appropriate measure of skill in the present context.

(G) *Breadth Of The Claims*

The instant claims focus on a limited universe of claimed core elements. The world of the instant claims is miniscule compared to the monoclonal antibody world approved for claiming in In re Wands, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988).

The Wands factors thus weigh in favor of the allowability of the present claims. Accordingly, reconsideration of the rejection under 35 U.S.C. §112, first paragraph is respectfully requested.

FEE DEFICIENCY

☒ If an extension of time is deemed required for consideration of this paper, please consider this paper to comprise a petition for such an extension of time; The Commissioner is hereby authorized to charge the fee for any such extension to Deposit Account No. 50-0258.

and/or

☒ If any additional fee is required for consideration of this paper, please charge Account No. 50-0258.

Closing Remarks

Applicant thanks the Examiner for the Office Action and believe this response to be a full and complete response to such Office Action. Accordingly, favorable reconsideration in view of this response and allowance of the pending claims are earnestly solicited.

Respectfully submitted,

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0022-1767/93/1511-6175$02.00/0
The Journal of Immunology
Printed in U.S.A.

Mapping the Major Human T Helper Epitopes of Tetanus Toxin

The Emerging Picture

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ABSTRACT. Progress on the mapping of Th epitopes of tetanus toxin (tt) has been slow due to reliance on studies of clones. In this paper, human Th cell epitopes of tt were mapped using proliferation tests on PBMC in response to synthetic peptides. PBMC from nine donors were tested over the entire set of tt homologous overlapping dodecapeptides. The 1304 peptides were initially tested as 66 pools, each containing an average of 20 peptides. PBMC from individual donors responded to as few as 1 and as many as 17 of the 66 peptide pools. The sequences responsible for proliferation were identified for the two most frequently recognized pools, and for another two pools within a major immunodominant region. Three new epitope sequences were mapped in detail and based on their recognition by most individuals are likely to be promiscuous. A cocktail of peptides including the newly identified Th cell epitopes was able to induce proliferation in PBMC from 24 of 31 tetanus toxoid (TT)-responsive donors. This cocktail is a chemically defined reagent that can be used to quantitate in vitro Ag-specific Th cells in PBMC from most subjects, and may thus be useful for serial measurements of specific immunity such as in subjects undergoing immunotherapy or immunosuppressive treatment. *Journal of Immunology,* 1993, 151: 6175.

TT[2] is commonly used in clinical or research studies of human T cell responsiveness as a control Ag or as a model Ag for studying Ag processing, presentation, and recognition mechanisms (1). Known epitopes of the untoxoided protein, tt, have been established by a combination of screening and predictive methods, largely by study of Th clones (2). A limitation of the methods used for initial location of determinant regions was that they relied on efficient processing of protein fragments by pathways similar to those operating with the whole Ag (1). It has been shown that cells deficient in specific enzymes can fail to process Ag and present a particular peptide, despite normal ability to process and present other peptides (3). Thus, use of long peptides or partially fragmented Ag could fail to reveal immunodominant regions of the Ag.

Frequencies of Th cells specific for TT can be very high in PBMC (4). It is therefore feasible to detect individual epitopes by direct stimulation of PBMC with peptides representing single epitopes, because precursor Th cells specific for single epitopes will be present in replicate samples of a donor's PBMC. We have found that use of pools of short synthetic peptides as Ag (J.C. Reece et al., manuscript in preparation) can allow epitope mapping with PBMC of any Ag of known sequence to which humans or animals have a strong Th response.

PBMC from donors shown to respond to TT in vitro were screened against peptide pools to locate the major epitopes in the sequence. The data obtained revealed five major epitopes, of which three had not been reported from studies using other approaches. The epitopes were then used, along with epitopes from other sources, in a survey of unselected donors to look for the breadth of recognition over a range of HLA types. The use of these materials as a chemically defined Ag for quantitation of Th cell responses in a clinical setting is proposed.

Materials and Methods

Medium

Complete medium consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, pH 7.4, and

Received for publication May 10, 1993. Accepted for publication August 20, 1993.

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[2] Abbreviations used in this paper: TT, tetanus toxoid; tt, tetanus toxin; b-dkp, beta-amino-alanine-diketopiperazine.

20 μg/ml gentamicin (CSL, Melbourne, Australia) to which 10% (vol/vol) human serum, pooled from donations screened for suitability in supporting in vitro PBMC proliferation, had been added.

Ag

Overlapping dodecapeptides for epitope scanning were synthesized by the multipin method (5) with a COOH-terminal b-dkp group and an acetylated NH_2-terminus. NH_2- and COOH-terminal-blocked peptides are as efficient in activation of Th cell clones as unblocked peptides (6, 7), in contrast to cytotoxic T cells (8). Peptides were cleaved into 0.1 M sodium bicarbonate in 96-well microtiter trays. The purity of representative peptides was assessed using HPLC and was found to be generally >80%. Wells were found to contain an average of 10 nmol cleaved peptide by amino acid analysis. Two independently synthesized sets of peptides made on pins were used for the final identification of T cell stimulatory dodecapeptides.

Bulk peptides for testing larger numbers of donors (Table VII) were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide synthesizer. Peptides were purified to >90% and their compositions were confirmed by amino acid analysis.

A cocktail of tt epitopes was prepared from equimolar amounts of peptides tt 141–171, 257–268, 591–602, 616–631, 640–651, 652–663, and 947–967. TT was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia.

Cell preparation

PBMC were from anticoagulated venous blood of healthy volunteers. PBMC were isolated by density interface centrifugation over Ficoll-Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The average yield of PBMC from whole blood was 2×10^6/ml with a range of 1.2×10^6/ml to 2.9×10^6/ml.

Standard PBMC proliferation assay

Peptide-stimulated proliferation assays using 2×10^5 PBMC per well were performed in 96-well round bottom microtiter plates (Nunc, Roskilde, Denmark). Ag were added in 20 μl of 0.1 M sodium bicarbonate to PBMC in complete medium to give a final volume of 200 μl per well. Assays were conducted using at least 16 replicates per test group. Cultures were incubated at 37°C in 5% CO_2 in humidified air. After 138 ± 2 h, proliferation was detected by pulsing with 0.5 μCi tritiated [*methyl*-^{3}H]thymidine (40 to 60 Ci/mmol, Amersham Australia, Sydney) per well for 6 h. Cells were harvested onto glass fiber filter mats (Skatron, Sterling, VA), and incorporated thymidine was measured using an LKB 1205 Betaplate liquid scintillation counter. All assays included at least 24 wells each of negative controls (20 μl of 0.1 M sodium bicarbonate instead of peptide solution) and positive controls (TT at 1.0 Lf/ml or 0.1 Lf/ml, also in 0.1 M sodium bicarbonate buffer).

MHC class II typing

MHC typing was performed on whole blood samples or EBV-transformed B cells by the Red Cross Blood Bank, Melbourne.

Method of statistical treatment of results

Data from large numbers of replicates per Ag-stimulated test group clearly demonstrated that the cpm values within a group are not normally distributed. This is a consequence of the random distribution of low numbers of specific responding T cells among the replicate wells. It is therefore inappropriate to treat proliferation data on PBMC by statistical methods based on normally distributed data. We have found the Poisson model is a better representation of the data (H.M. Geysen et al., manuscript in preparation) and therefore chose to use the following method. A cutoff was calculated in the conventional way assuming that data from the unstimulated (cells alone or no Ag negative control) group was normally distributed. A cutoff cpm value of the mean plus three times the SD of the cells alone group was calculated and used to score each well as negative (below the cutoff) or positive. Poisson statistics were used to determine whether any difference in the numbers of positive wells between the negative control (cells alone) group and each experimental group was significant. Frequencies of positive responses significant at the 0.25% or better ($p < 0.0025$) level are reported.

Because this method of analysis is uncommon for proliferation tests but common in other quantal methods, we have included a typical set of data from the pools scan of one donor comparing this method of analysis with a conventional method using the mean ± SD of the ^{3}H-TdR uptake (cpm) (Fig. 1). Figure 1*A* shows that for several peptide tests, the mean is higher than the mean of the cells alone but the SD is generally large so a simple statistical test will not distinguish any test groups from the negative control group. This is a direct result of the low frequency of peptide-specific Th cells: only wells with peptide-specific Th cells can show proliferation. In contrast (Fig. 1*B*), classifying each well as either proliferating or nonproliferating and using a statistical test to distinguish groups significantly different from the cells alone according to the frequency of wells displaying proliferation is logical and objective. Figure 1 also gives an indication of the magnitude of peptide responses and generally shows the higher the mean, the greater the frequency of positive wells, as expected. We assigned a cutoff of the mean + 3SD based on the assumption of normally distributed background cpm, and it is evident that there are borderline cases between

proliferating wells and nonproliferating wells (Fig. 1). This is inevitable where there is a continuous spectrum of values.

Results

Peptide pooling strategy

A set of 1304 overlapping 12mer peptides was synthesized spanning the 1315 residues of the tt sequence (9), each peptide offset by one residue from the preceding peptide. Thus, each peptide overlapped the preceding and the following one by 11 residues. The multipin peptide synthesis system used gave nontoxic peptide solutions ready for use in bioassays. Because it was impractical to screen each peptide separately on each donor's PBMC for its ability to cause proliferation, we used a peptide pooling strategy to identify regions containing Th cell epitopes, followed by testing of individual peptides from the most frequently recognized pools.

We chose to screen peptides as 66 pools of approximately 20 sequential overlapping peptides each (Table I). The size of the pools was selected so that the size of both the initial scan and the subsequent decodes of stimulatory pools would be manageable. Due to the completeness of the peptide set, the peptides from the NH_2-terminal end of a pool overlap with the preceding pool and likewise the peptides from the COOH-terminal end of a pool overlap with the following pool.

The concentration of each peptide used in the final culture was 0.3 μM. Epitopes of less than 12 residues in length will be present in two, three, or more of the overlapping peptides in that pool, and therefore the concentration of shorter epitopes will be higher than that of longer epitopes.

PBMC from nine HLA-typed donors (Table II), known to respond to TT in vitro, were initially scanned for their ability to respond to each of the 66 peptide pools (Table I). Peptide pool/donor combinations scored as positive are those in which proliferation occurred in a significantly larger number of wells than seen in the cells alone control ($p < 0.0025$). Figure 1 shows a typical set of data from the pool scan of one donor comparing this method of analysis with the conventional method of using the mean ± SD.

Table I shows that several pools stimulated PBMC from

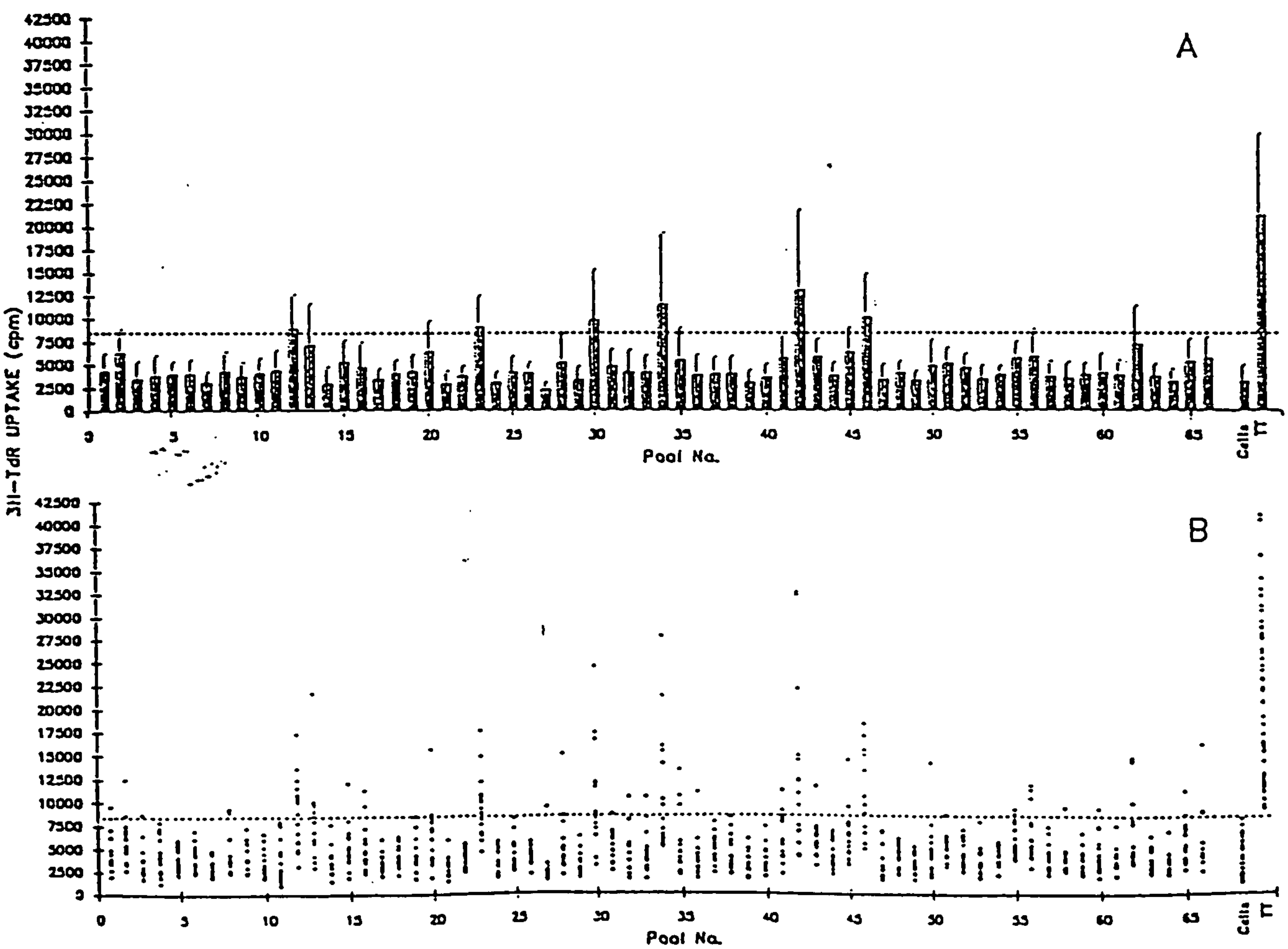


FIGURE 1. The data from the tt pools scan using donor H from Table I. *A*: The mean ± SD of incorporated [^{3}H]-TdR (cpm). *B*: A scatter plot of the incorporated [^{3}H]-TdR (cpm) of individual wells.

Table I
Complete scan of tt for Th cell epitopes using peptide pools and PBMC

Pool[a] No.	tt Sequence Spanned by Pool	Donor A	B	C	D	E	F	G	H	I	No. +VE Donors
1	1 to 31										
2	21 to 51							+[b]			+
3*	41 to 69										
4	59 to 89										
5	79 to 109	+									+
6	99 to 129										
7	119 to 149							+			+
8	139 to 169					+	+			+	+++
9	159 to 189						+			+	++
10	179 to 209										
11	199 to 229		+					+		+	+++
12	219 to 249					+			+		++
13	239 to 269							+	+		++
14	259 to 289										
15	279 to 309									+	+
16	299 to 329										
17	319 to 349										
18	339 to 369										
19	359 to 389										
20	379 to 409								+		+
21	399 to 429	+									+
22	419 to 449							+			+
23	439 to 469								+		+
24	459 to 489										
25	479 to 509										
26	499 to 529					+		+			++
27	519 to 549										
28	539 to 569					+					+
29	559 to 589										
30	579 to 609		+			+	+		+	+	+++++
31	599 to 629		+		+						++
32	619 to 649					+				+	++
33	639 to 669					+	+			+	+++
34	659 to 689		+						+		++
35	679 to 709								+		+
36	699 to 729										
37	719 to 749			+			+			+	+++
38	739 to 769									+	+
39	759 to 789										
40	779 to 809										
41	799 to 829		+							+	++
42*	819 to 847					+	+		+	+	++++
43	837 to 867		+				+				++
44	857 to 887										
45	877 to 907										
46	897 to 927								+	+	++
47	917 to 947										
48	937 to 967										
49	957 to 987						+			+	++
50	977 to 1007						+				+
51	997 to 1027										
52	1017 to 1047										
53	1037 to 1067										
54	1057 to 1087										
55	1077 to 1107										
56	1097 to 1127						+		+	+	+++
57	1117 to 1147										
58	1137 to 1167						+				+
59	1157 to 1187						+			+	++
60	1177 to 1207					+	+			+	+++
61	1197 to 1227										
62	1217 to 1247		+				+		+		+++
63	1237 to 1267									+	+
64	1257 to 1287					+					+
65	1277 to 1307										
66*	1297 to 1315		+					+	+		+++
Negative control		1/112	3/112	0/112	1/80	1/112	3/112	3/112	1/112	2/112	
Positive control (TT)		49/56	38/56	26/56	25/40	14/56	16/56	44/56	56/56	56/56	

[a] Each peptide pool consisted of 20 overlapping 12mer peptides unless specified by an *.
[b] Pools scored positive ($p < 0.0025$) using 16 replicates per test.

Table II
Map of class II MHC Ag for donors used in pooling scan of tt

Donor	HLA Typing					
	DR	DR	DRW	DRW	DQ	DQ
	11	13	52		1	7
B	2	4		53	1	7
C	4	7		53	7	9
D	4	7		53	2	8
E	2	3	52		1	2
F	2				1	
G	4	13	52	53	1	8
H	1	7		53	1	9
I	1	2			1	

...re than one donor. Two major areas of reactivity were ...ols 30 (tt residues 579–609) and 42 (tt residues 819–847). A further eight pools stimulated PBMC from one-third (3/9) of the donors. Six of the nine donors responded to a pool unique for that donor, whereas 29 of 66 (44%) of the pools were not shown to be stimulatory for any of the donors tested under the stringent criterion used ($p < 0.0025$).

Location of Th cell epitopes within stimulatory pools

The individual peptides within four stimulatory pools were tested to identify the peptide(s) responsible for proliferative responses incurred by the pool. For convenience, we call this test a decode. Single peptides were tested at 1 μM, approximately three times the concentration of individual peptides used in the pool. This was because when more than one peptide within a pool contains an epitope, the effective concentration is proportionally higher. For example, if an epitope for a single Th cell consisted of nine amino acids (10, 11), stimulatory sequences would be present in four consecutive overlapping peptides within the pool, making the effective concentration of that epitope 1.2 μM.

Decoding of the most commonly recognized pools, 30 and 42 (Tables III and IV, respectively), enabled us to see whether published Th cell epitopes would be precisely identified using this method. Peptides within pool 30 contain sequence YSYFPSVI (tt 593–600), the epitope for a human tt-specific Th cell clone (10). Decoding of pool 30 showed that five overlapping 12mers with start residues 589 to 593 were stimulatory for at least one of the three donors (Table III). These 12mers all contain the sequence YSYFPSVI, identical to the published epitope (10, 12).

Pool 42 spans sequence QYIKANSKFIGITEL (tt 830–844), reported to contain a universally immunogenic DR-restricted epitope (1). Decoding of pool 42 showed that five 12mers with start residues between 827 to 831 were capable of stimulating PBMC from four donors tested (Table IV). All these peptides overlap a core (6) of eight residues, YIKANSKF, within the reported epitope tt 830–844 (1).

Because the region tt 579–689 (pools 30 to 34) consisted of five commonly stimulatory pools, we chose to decode two additional pools within this region to identify epitopes not previously reported. Testing of individual peptides within pool 33 on four donors' cells showed that the response to this pool was due to two distinct determinant regions (Table V). Donor B, although not scored positive for this pool in Table I, had shown responses to pool 33 at the less stringent level of $p < 0.05$, and was thus included in the testing on single peptides of pool 33 (Table V). The Th cell epitopes within this region were centered on sequences IVPYIGPA (tt 642–649) and KQGYEGNFI (tt 654–662), respectively.

Decoding of pool 31 and the first two peptides of pool 32 revealed with donor D a series of six overlapping stimulatory 12mers with start residues 616 to 620 (Table VI). All these peptides contain the 7mer core sequence IDDFTNE (tt 620–626). Donors B and G responded to one and two peptides, respectively, containing the core sequence (Table VI). Donor G was included in the testing of single peptides within pool 31 because in the tt pools scan positive responses to pool 31 were significant at the $p < 0.05$ level.

We sought to determine whether these findings using 12mer peptides would also apply for longer peptides. We tested the ability of a 16mer, which encompassed the "envelope" sequence (6) of the stimulatory peptides from pool 31, to stimulate PBMC of ~~donors B and D~~ and a random set of donors (Table VII) (residues 616 to 631). More than half of the donors responded to this 16mer, implying that it is a "promiscuous" epitope.

616- TT4

The other two newly identified T cell determinants (Tables V and VI) and four other peptides containing T cell epitopes of tt were also tested on the random set of donors (see footnote to Table VII). Of the four other tt peptides, two were identified in experiments conducted concurrently with the work reported herein, i.e., tt 141–171, corresponding closely to pool 8 (Table I), and tt 257–268 which was found using an unpublished algorithm (data not shown) but was associated with only two responders in the original scan of nine donors (Table I, pool 13).

In the survey of 32 additional donors (Table VII), peptides were tested at two concentrations, 10 and 1 μM, using 32 replicates per test. The 31-residue tt 141–171 and the 12mer tt 640–651 (Table VII) gave highly significant ($p < 0.0025$) responses at one or both peptide concentrations in at least half of the donors. All of the remaining peptides, including the promiscuous epitope tt 947–967 (1), stimulated PBMC of at least one quarter of the donors. One donor who did not respond to TT in vitro also failed to respond to any of the tt peptides, despite being responsive to other peptide and control Ag (data not shown). This suggests Ag specificity of the responses to tt peptides, which was also suggested in restimulation experiments and studies on immunization of volunteers with TT (J.C. Reece et al., manuscript in preparation).

TT5

Three donors responded well to TT but not to any of the

Table III
Decode of stimulatory pool 30 spanning residues of tt 579–609

Peptide Start No.	Sequence	Donor		
		B	F	I
579	TNSVOOALINST[a]	—	—	—
580	NSVOOALINSTK	—	—	—
581	SVOOALINSTKI	—	—	—
582	VOOALINSTKIY	—	—	—
583	OOALINSTKIYS	—	—	—
584	OALINSTKIYSY	—	—	—
585	ALINSTKIYSYF	—	—	—
586	LINSTKIYSYFP	—	—	—
587	INSTKIYSYFPS	—	—	—
588	NSTKIYSYFPSV	—	—	—
589	STKIYSYFPSVI	—	—	3[b]
590	TKIYSYFPSVIS	4	3	—
591	KIYSYFPSVISK	—	3	5
592	IYSYFPSVISKV	4	3	4
593	YSYFPSVISKVN	—	4	—
594	SYFPSVISKVNQ	—	—	—
595	YFPSVISKVNQG	—	—	—
596	FPSVISKVNQGA	—	—	—
597	PSVISKVNQGAQ	—	—	—
598	SVISKVNQGAQG	—	—	—
Cells alone		1/72[c]	2/96	3/120
Pool 30		5/16	6/16	5/16
TT 0.1 Lf/ml		36/40	34/56	55/56

[a] Individual peptides were tested at a concentration of 1 μM.
[b] Number of positive wells out of 16 replicate wells. Only frequencies of positive wells that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from the cells alone control ($p > 0.0025$).
[c] Number of positive wells out of the number of wells shown.

peptides. These results indicate that these tt T cell epitopes display at least partial MHC class II restriction (Table VII). These results also show that the peptides do not exhibit nonspecific mitogenic activity.

Figure 2 summarizes the major human Th cell epitopes of tt, both from this study and from published data, with emphasis placed on those epitopes known or likely to be promiscuous.

Testing a cocktail of dominant human tt Th cell epitopes

To determine whether a cocktail of dominant epitopes of an Ag could be used as a chemically defined reagent in place of the whole Ag, seven Th cell epitopes of tt were pooled together and tested in parallel with TT (Table VII). The cocktail comprised previously reported epitopes (1, 12) and epitopes identified by this study (*Materials and Methods*). The seven tt peptides were tested individually to identify the peptide(s) responsible for responses incurred by the cocktail (Table VII).

As expected, the frequency of positive wells was generally as high as the strongest of the individual peptide frequencies (Table VII). A higher proportion of TT-immune donors (24 of 31) responded to the pool than to any individual peptide. These results confirm that the cocktail used does not have an epitope for all donors, but show that combining epitopes is a practical way to create a chemically defined T cell stimulatory reagent for studies on PBMC.

Discussion

Many Th cell determinant regions of tt were identified, and four of these were examined in detail, resulting in the mapping of a total of five epitopes. The most frequently recognized sequence corresponds to a published Th epitope for a single human T cell clone (10, 12), whereas another corresponds to a published promiscuous Th cell epitope (1). Reliance on predictive methods or on screening of T cell clones for epitope specificity had not previously identified three of these epitopes. These results allow a map of human Th cell tt epitopes to be drawn (Fig. 2). There are clearly further sites to be decoded in detail (Table I) to build up a more complete map.

The success of this approach in identifying epitopes with PBMC may stem from the use of short peptides. Protein cleavage fragments (2) or long synthetic peptides with small overlaps (14) may fail to stimulate Th cells to proliferate, due to cleavage of epitope sites or inappropriate processing of peptide (J.C. Reece et al., manuscript in preparation). Use of all overlapping peptides of a length within the range of naturally processed peptides (13 to 18 residues) (15, 16) can result in presentation of the specified epitope without the need for processing. With the pooling/

Table IV
Decode of stimulatory pool 42 spanning residues of tt 819–847

Peptide Start No.	Sequence	Donor			
		E	F	H	I
819	EFDTOSKNILMQ[a]	—	—	—	—
820	FDTOSKNILMOY	—	—	—	—
821	DTOSKNILMOYI	—	—	—	—
822	TOSKNILMOYIK	—	—	—	—
823	OSKNILMOYIKA	—	—	—	—
824	SKNILMOYIKAN	—	—	—	—
825	KNILMOYIKANS	—	—	—	—
826	NILMOYIKANSK	—	—	—	—
827	ILMOYIKANSKF	7[b]	—	—	—
828	LMOYIKANSKFI	6	—	6	5
829	MOYIKANSKFIG	5	—	14	—
830	OYIKANSKFIGI	4	—	5	—
831	YIKANSKFIGIT	—	3	—	—
832	IKANSKFIGITE	—	—	—	—
833	KANSKFIGITEL	—	—	—	—
834	ANSKFIGITELK	—	—	—	—
835	NSKFIGITELKK	—	—	—	—
836	SKFIGITELKKL	—	—	—	—
Cells alone		2/88[c]	8/252	3/100	1/60
Pool 42		5/24	[d]	4/24	[d]
TT 0.1 Lf/ml		24/24	24/24	24/24	16/16

[a] Individual peptides were tested at a concentration of 1 μM.
[b] Number of positive wells out of 24 replicate wells. Only frequencies of positive wells that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from the cells alone control ($p > 0.0025$).
[c] Number of positive wells out of the number of wells shown.
[d] Donors F and I were shown to respond to pool 42 in the initial pool scanning assay (Table I).

Table V
Decode of stimulatory pool 33 and the last peptide of pool 32

Peptide Start No.	Sequence	Donor			
		B	E	F	I
638	DVSTIVPYIGPA[a]	•[b]	•	8[c]	—
639	VSTIVPYIGPAL	7	4	•	•
640	STIVPYIGPALN	4	4	•	•
641	TIVPYIGPALNI	6	3	15	10
642	IVPYIGPALNIV	—	—	4	3
643	VPYIGPALNIVK	—	—	—	—
644	PYIGPALNIVKO	—	—	—	—
645	YIGPALNIVKOG	—	—	—	—
646	IGPALNIVKOGY	—	—	—	—
647	GPALNIVKOGYE	—	—	—	—
648	PALNIVKOGYEG	—	—	—	—
649	ALNIVKOGYEGN	—	—	—	—
650	LNIVKOGYEGNF	—	—	—	—
651	NIVKOGYEGNFI	—	—	10	—
652	IVKOGYEGNFIG	8	2	—	—
653	VKOGYEGNFIGA	—	—	—	—
654	KOGYEGNFIGAL	—	—	—	—
655	OGYEGNFIGALE	—	—	—	—
656	GYEGNFIGALET	—	—	—	—
657	YEGNFIGALETT	—	—	—	—
658	EGNFIGALETTG	—	—	—	—
Cells alone		1/63[d]	3/132	2/77	2/77
Pool 33		23/24	16/24	24/24	16/24
TT 0.1 Lf/ml		23/24	13/24	19/24	24/24

[a] Individual peptides were tested at a concentration of 1 μM.
[b] Not tested.
[c] Number of positive wells out of 16 replicate wells. Only frequencies that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from cells alone ($p > 0.0025$).
[d] Number of positive wells out of the number of wells shown.

TT5

p638–653
DVSTIVPYIGPALNIV

amount of sequence needed to allow MHC class II binding and recognition by the TCR.

Even though the 12mers detected many previously unknown epitopes, had we used longer peptides we may have detected more determinant regions. For example, individuals frequently respond to tt 947–967 (Table VII) but not to shorter peptides spanning this region (Table I, and additional data not shown). Thus, this new map, although more thorough than any previously reported, is only a first step toward the full set of epitopes for tt. The donors also represent a limited spectrum of MHC types, ensuring that there are further epitopes presented by other allotypes yet to be defined.

Within the four pools decoded, there were cases where at least six overlapping 12mer peptides were stimulatory. The proliferative response to these related peptides could be due to the activation of clonal progeny of one precursor T cell by a sequence common to the peptides. Alternatively, these observations may result from activation of a number of independent T cell clones able to respond to different but overlapping sequences. The reported finding that the NH_2-terminus of the peptide was an important and consistent part of the peptide that binds to MHC class II Ag (15) suggested

decoding approach, the otherwise daunting task of testing all these short peptides of an Ag on PBMC of individual donors is achievable.

We have found that the peptide pooling strategy using human PBMC works well for identifying the Th cell epitopes within Ag from influenza, allergens, and HIV-1 (data not shown). The frequencies of Ag-specific Th cells for some of these Ag were generally lower than for TT indicating that it is not necessary to choose Ag with exceptionally high frequencies of Ag-specific Th cells. In addition, we have found that the peptide pooling strategy can be applied to epitope mapping with spleen and lymph node cells from animals as well as to PBMC (data not shown).

The physical length of the peptides used herein is consistent with the 13 to 18 residue length range of the peptides naturally bound to class II Ag (15, 16). This is because all peptides used in the pools contain 12 residues of the tt sequence with a constant tripeptide moiety (b-dkp) (5) at the COOH-terminal end and an acetyl group at the NH_2-terminal end. An acetylated NH_2-terminus can lead to increased effectiveness of Th epitope peptides (7). Th cell clones can be stimulated by b-dkp-bearing peptides of 8, 9, or 10 residues (10) suggesting that peptides containing 12 residues of the Ag sequence have more than the required

Table VI
Decode of pool 31 and the first two peptides of pool 32

Peptide Start No.	Sequence	Donor		
		B	D	G[a]
599	VISKVNOGAOGI[b]	—	—	—
600	ISKVNOGAOGIL	—	—	—
601	SKVNOGAOGILF	—	—	—
602	KVNOGAOGILFL	—	—	—
603	VNOGAOGILFLO	—	—	—
604	NOGAOGILFLOW	—	—	—
605	OGAOGILFLOWV	—	—	—
606	GAOGILFLOWVR	—	—	—
607	AOGILFLOWVRO	—	—	—
608	OGILFLOWVROI	—	—	—
609	GILFLOWVROII	—	—	—
610	ILFLOWVROIIO	—	—	—
611	LFLOWVROIIOO	—	—	—
612	FLOWVROIIOOF	—	—	—
613	LOWVRDIIOOFT	—	—	—
614	OWVROIIDOFTN	—	—	—
615	WVROIIOOFTNE	—	8[c]	—
616	VROIIOOFTNES	—	8	—
617	ROIIOOFTNESS	—	8	2
618	OIIOOFTNESSO	—	12	•[d]
619	IIOOFTNESSOK	7	15	3
620	IOOFTNESSOKT	—	4	—
Cells alone		1/108[e]	2/104	5/216
Pool 31		6/24	16/24	3/24
TT 0.1 Lf/ml		19/20	24/24	24/24

[a] Donor G gave significant proliferative responses at the $p < 0.05$ level.
[b] Individual peptides were tested at a concentration of 1 μM.
[c] Number of positive wells out of 24 replicate wells. Only frequencies of positive wells that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from the cells alone control ($p > 0.0025$).
[d] Not tested.
[e] Number of positive wells out of the number of wells shown.

TT4

p615–631 = WVRDIIDDFTNESSQKT

Table VII
Summary of PBMC responses to tt peptides and peptide cocktail

Donor	Peptide 141–171[a] 10 µM	1 µM	257–268[b] 10 µM	1 µM	591–602[c] 10 µM	1 µM	616–631[d] 10 µM	1 µM	640–651[e] 10 µM	1 µM	652–663[e] 10 µM	1 µM	947–967[f] 1 µM[h]	Cocktail[g] 1 µM	TT 0.5 Lf/ml	Cells Alone
B	NT	NT[i]	NT	NT	NT	NT	9	3[j]	NT	NT	NT	NT	NT	NT	24/24	0/48
D	NT	NT	NT	NT	NT	NT	29	21	NT	NT	NT	NT	NT	NT	24/24	5/82
CM1	32	32	31	25	4	27	13	5	32	32	7	6	23	32	24/24	1/102
CM2	—	—[k]	—	—	—	—	16	17	—	—	8	—	—	24	24/24	3/102
CM3	—	—	32	32	30	19	28	—	29	—	16	11	5	32	24/24	2/95
CM4	—	—	—	—	—	—	—	—	—	—	—	—	—	8	24/24	2/102
CM5	7	9	17	14	—	—	—	—	17	10	17	—	—	31	24/24	1/102
CM6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	18/18	1/45
CM7	—	—	8	7	—	—	4	—	7	—	4	—	28	31	24/24	1/102
CM8	30	32	—	—	15	—	4	—	24	15	—	—	9	31	24/24	1/102
CM9	13	12	—	—	—	—	—	—	—	—	—	—	—	11	23/24	1/32
CM10	14	16	10	16	NT	NT	19	14	NT	NT	24	NT	7	NT	16/18	0/74
CM11	—	—	28	25	27	23	15	—	10	—	25	10	—	32	24/24	2/140
CM12	—	—	—	—	—	—	—	—	24	—	5	—	—	—	18/18	1/68
CM13	—	—	—	—	6	—	—	—	—	—	—	—	—	26	24/24	2/102
RX45	—	—	—	—	—	—	29	32	10	—	—	—	—	29	24/24	2/102
RX46	31	32	14	22	32	25	32	32	32	32	32	20	31	32	24/24	1/102
RX47	22	8	—	—	—	—	14	8	23	9	—	—	—	20	NT	1/36
RX48	—	—	9	14	13	15	13	5	5	—	10	5	10	28	24/24	2/198
RX49	—	—	—	—	5	—	27	28	5	—	—	—	—	21	15/15	0/38
RX50	9	5	—	—	—	—	—	—	7	—	—	—	—	10	17/24	4/152
RX51	29	18	7	—	14	—	7	—	32	25	18	9	8	26	24/24	2/101
RX53	20	27	—	—	—	7	25	29	24	29	6	5	7	31	24/24	2/140
RX54	8	15	—	—	—	—	—	—	4	8	—	—	4	16	8/24	3/140
RX55	6	3	5	4	—	—	3	—	—	—	—	—	3	15	24/24	0/102
RX56	31	17	—	—	—	—	—	—	3	—	—	—	—	32	24/24	0/102
RX57	6	—	—	—	—	—	—	—	—	—	—	—	—	—	10/24	0/98
RX59	—	—	—	—	—	—	—	—	—	—	—	—	—	—	18/24	2/102
RX60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	24/24	2/102
RX61	13	7	4	5	—	—	—	—	8	7	12	—	—	17	22/24	1/102
RX62	—	—	—	—	—	—	—	—	—	—	—	—	6	—	24/24	1/102
RX63	—	—	—	—	—	—	—	—	—	—	—	8	—	—	23/24	3/102
RX64	—	—	7	—	—	—	13	—	—	—	—	—	—	30	24/24	1/102
RX65	5	—	5	—	—	—	4	—	—	—	—	—	—	7	24/24	1/102
+VE donors/ Total	16/32		13/32		10/31		19/34		18/31		14/32		12/32	24/31	33/33	

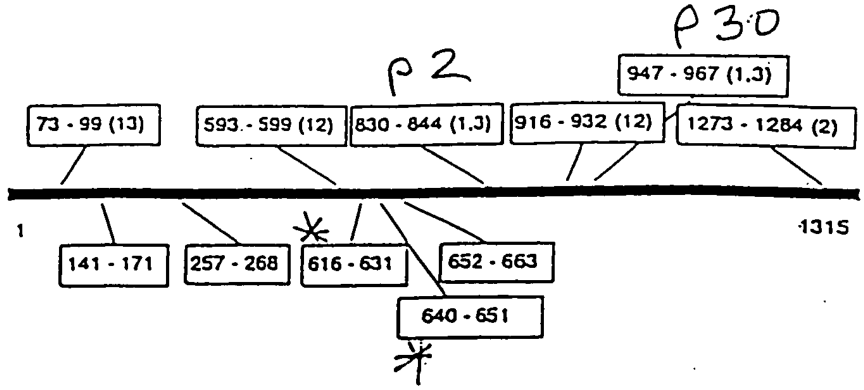
P30

[a] Peptide identified from pooling scan (pool 8).
[b] Peptide identified by predictive algorithm.
[c] 12mer peptide identified by decoding pool 30.
[d] 16mer peptide identified by decoding of pool 31.
[e] 12mer peptide identified by decoding pool 33.
[f] 21mer peptide identified by Panina-Bordignon (refs. 4, 11).
[g] Cocktail consists of seven individual peptides each at 1 µM.
[h] 947–967 was shown to be cytotoxic at 10 µM so it was only tested at 1 µM.
[i] Not tested.
[j] Number of wells scored positive ($p < 0.0025$) using 32 replicate wells.
[k] "—" denotes not significantly different from cells alone control ($p > 0.0025$).

that peptides differing in NH_2-terminal position by only one residue would activate different populations of Th cells. If this is the case, the prediction would be that testing smaller numbers of longer peptides could result in failure to detect some epitopes, because peptides with the required NH_2-terminal residues may not be present in the pool. Because overlapping peptides (Tables III to VI) are stimulatory, it is probably not critical to have a particular NH_2-terminal residue to successfully map most epitopes with PBMC. A study on a human tt-specific Th clone (10) found that the NH_2-terminal residue of the minimum stimulatory peptide was the most replaceable amino acid, whereas the COOH-terminal residue could only be changed from I to L, suggesting that the NH_2-terminal residue is less significant than implied from peptide isolation studies (15).

APC play a critical role in Ag-stimulated PBMC proliferation assays. Short synthetic peptides can be efficiently presented by a range of APC, including B cells, monocytes and dendritic cells (17). It is known that short peptides can be taken up directly by MHC class II molecules without being processed (18, 19) but the relative significance of this pathway vs an intracellular pathway for peptides presented by APC in PBMC is unknown at this time (20). For longe

FIGURE 2. A map of the major hum·· Th epitopes of tt. On a linear sc. , the known epitopes are indicated above the line representing the 1315 residues of tt and the newly identified epitopes are indicated below the line.



:ides, however, inefficient detection of precursor T cells may be occurring because certain pools of 12mers were stimulatory for PBMC, whereas 31mer peptides spanning the same sequences as the stimulatory pools were not (J.C. Reece et al., manuscript in preparation).

Because we expected the quantitative response of PBMC to be dependent on peptide concentration, we included two dose levels in the survey of seven epitopes (Table VII). We chose to treat significant responses ($p < 0.0025$) at either dose level as representing recognition of an epitope. Although 10 μM often gave higher frequencies of responding wells, there are many instances of the opposite effect, suggesting that this concentration range is a good compromise for most of the peptide/donor combinations.

Better knowledge of the immunodominant and promiscuous epitopes of Ag as determined from unselected Th cells will allow design of reagents for enhancement of immunogenicity of Ag (e.g., vaccines) in humans. Such reagents may be of more general applicability than those established from study of the best-growing clones (21). If antagonistic peptides that have the potential to alleviate autoimmune disease are to be practical (22, 23), the epitopes responsible for disease need to be rapidly located for a spectrum of MHC allotypes without the lengthy and laborious establishment and characterization of clones. This study shows that this can be done for a large Ag.

A cocktail of T cell epitopes may be an effective substitute for whole Ag in diagnostic assays for Th cell function. In the few cases where there was no measurable response to the tt cocktail (Table VII), the responses to individual peptides were seen at only one concentration and were generally low. This cocktail of peptides thus represents a synthetic T cell stimulatory Ag that could be used to standardize T cell proliferation tests on most TT-immune subjects. Serial monitoring of PBMC responses would not be subject to the uncertainty of batch variation in TT or variations in the effective dose of presented peptide.

The identification of the whole spectrum of Th cell epitopes may allow a greater understanding of the basis of epitope selection for MHC class II-restricted epitopes. This may enable accurate prediction of Th cell epitopes from primary sequence data alone.

Acknowledgments

We thank Richard Lauricella, Megan Jay, and the peptide synthesis team for synthesis of the peptides, and the many willing blood donors. We also thank Dr. Gordon Whyte of the Red Cross Blood Bank, Melbourne, for the supply of buffy coats.

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Synthesis of Multiple Peptides on Plastic Pins

Scanning protein sequences by bioassay for smaller bioactive peptide sequences requires a source of many peptides homologous with the parent protein sequence. This unit deals with one of the synthetic methods for making such sets of peptides (see Fig. 9.7.1). The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale. The multipin technology is suitable because it can be performed without expensive synthesizers and it uses equipment available to most laboratories. Prior experience with organic synthesis techniques or peptide chemistry is useful but not essential. The products of synthesis by multipin technology are unpurified peptides which are useful as screening reagents and may also be used to prepare purified peptide on a small scale.

Most multipin techniques exploit the conventional 8 × 12 matrix layout of common microtiter equipment which simplifies handling of the synthesis, the products (peptides), and the test results. Computer assistance with synthesis and data analysis also speeds the cycle from designing the experiment through analyzing the results.

With multipin technology, peptides are synthesized in parallel on plastic "pins" (Fig. 9.7.2) to give sets of peptides suitable not only for B and T cell epitope scanning but also for other bioassays. Peptides can be either permanently bound to the surface of the plastic for direct binding assays, or they can be released into solution. There is a choice of N- and C-terminal peptide endings. For solution-phase peptides, the synthesis scale can be 1 or 5 μmol (for a 10-mer, ~1 mg or 5 mg, respectively). The preferred coupling/deprotection chemistry used is the milder 9-fluorenylmethyloxycarbonyl (Fmoc) protection scheme rather than the older *t*-butyloxycarbonyl (*t*-Boc) protection scheme (see *UNIT 9.1*), thus reducing the level of chemical safety risk arising from synthetic peptide chemistry.

This unit covers the strategy of the multiple peptide approach to biological scanning, the synthetic protocols, and the handling of peptides after synthesis—cleavage, preliminary purification, storage, and analysis (see Basic Protocol). It is specific for the multipin technique using equipment obtained from Chiron Technologies, although some of the approaches are applicable to other multiple synthesis techniques. Procedures for multipin equipment obtained from other suppliers may differ from the procedures described here, and the manufacturer's literature should be consulted. This unit also includes protocols for preparing Fmoc-amino acid solutions (see Support Protocol 1) and for acetylating (see Support Protocol 2) or biotinylating (see Support Protocol 3) synthesized peptides.

STRATEGIC PLANNING

For a protein whose primary structure is known, the conceptually simplest method of locating all the bioactive linear peptide sequences is to make all possible peptide subsets of the protein sequence and test them. If only selected parts of the sequence are synthesized, or only the predicted active parts, bioactive sequences could be missed. The use of a set of highly overlapping peptides likewise reduces the possibility that the most bioactive sequences might be missed because they are absent from the set. For example, a set of all overlapping 20-mers offset along the sequence by one residue at a time should capture the entire set of helper T cell epitopes, and this is a much more reliable approach than trying to predict motifs. In reality, a synthetic peptide scan through a protein is a compromise between the cost and effort in making and screening all peptides and the need for completeness. Thus, one worker may choose to make all overlapping 8-mers to

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Current Protocols in Immunology (1997) 9.7.1-9.7.19

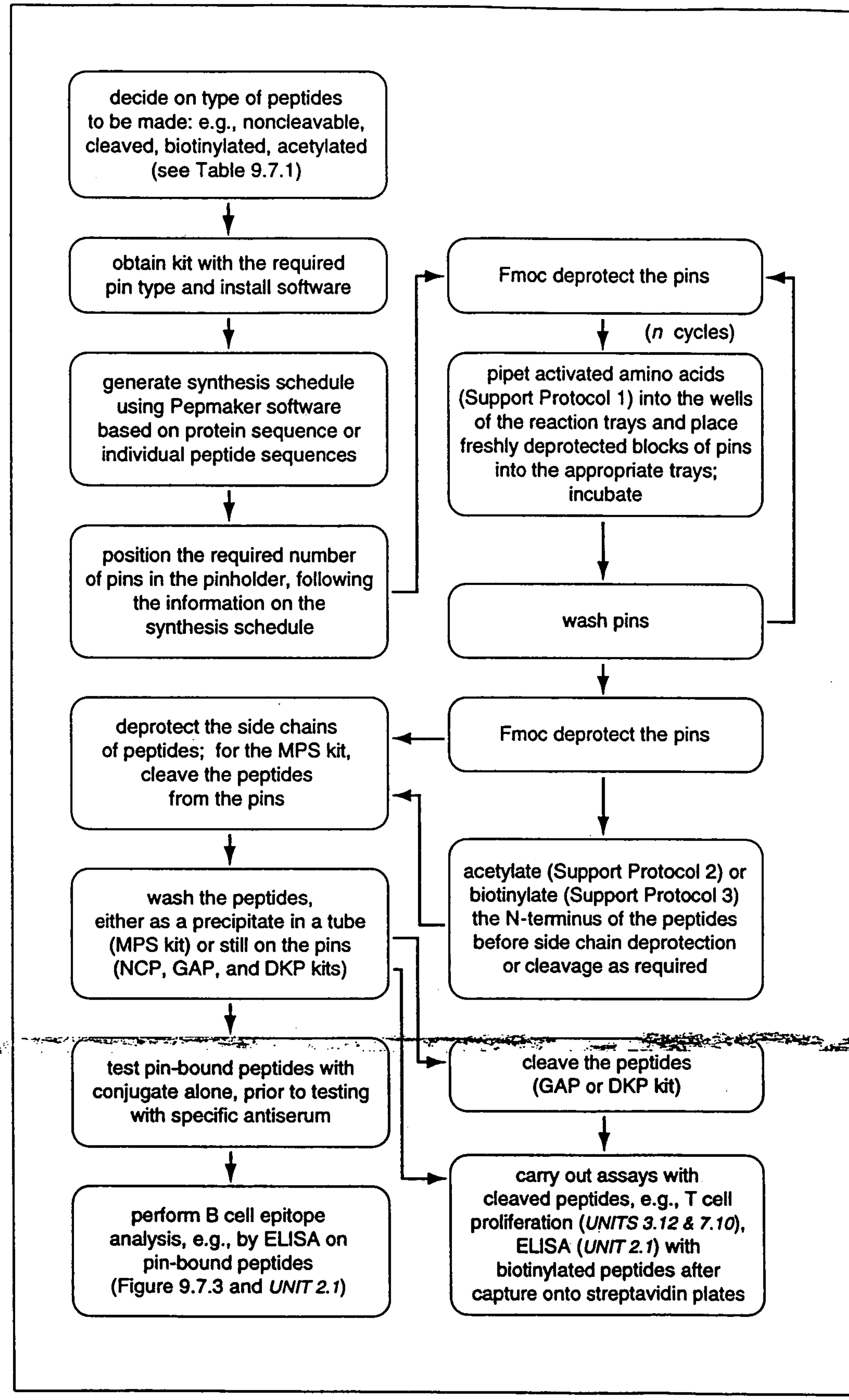

Figure 9.7.1 Flow chart for multipin peptide synthesis.

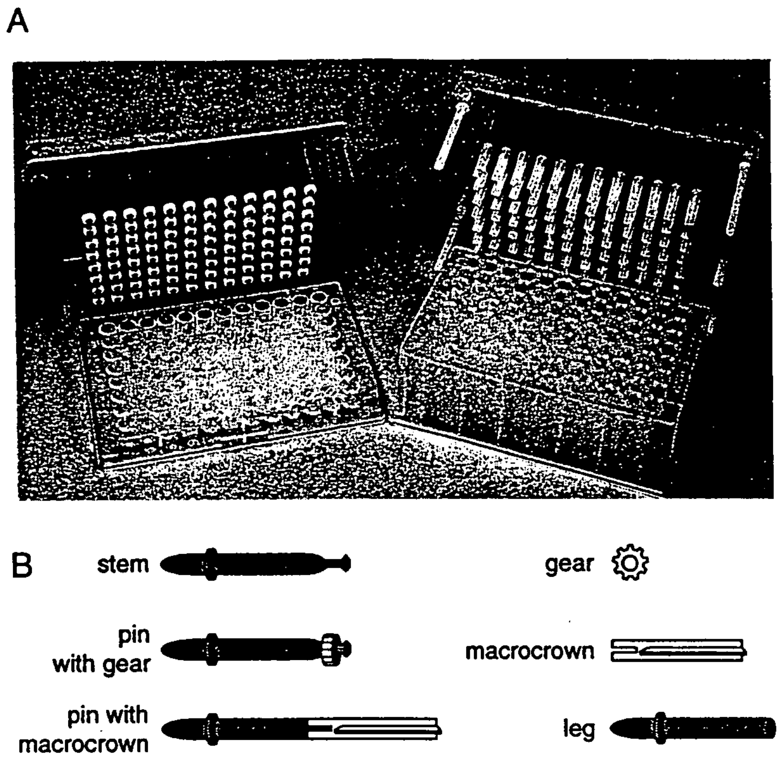

Figure 9.7.2 Apparatus for multipin peptide synthesis. **(A)** Assembled synthesis block with 96 gears (left) or 96 macrocrowns (right). **(B)** Components of the pin assembly. Components are either push-fitted together (e.g., legs or stems into the pin holder) or clipped on (gears or macrocrowns onto stems). All components are solvent-resistant plastic, either polyethylene, polypropylene, or copolymers of these two monomer types.

find the linear (continuous) B cell epitopes, and another may make 12-mers offset along the sequence by five residues for the same purpose. In each case, all sequences of eight residues from the protein are present in at least one peptide, but the latter approach requires only one-fifth the number of peptides.

Planning the Synthesis

Synthetic peptides are assembled by solid-phase synthesis one amino acid at a time, commencing with the C-terminal end of the peptide on the solid phase (see *UNIT 9.1*).

The assembly process, or coupling, requires activation of the α-carboxyl group of each incoming amino acid to make it reactive with the α-amino group of the growing peptide chain. To prevent unwanted polymerization or side reaction, reactive groups in each amino acid must be temporarily protected, and the protecting group removed before further reaction can be carried out. The protecting group on the α-amino function of the most recently added amino acid must be removed before another amino acid can be coupled to it, so the α-amino protection must be labile under conditions that do not remove side-chain protection. Later, the side-chain-protecting groups must be removable under conditions that do not attack the peptide bonds. The two common protecting group "schemes" are known as *t*-butoxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The protecting group scheme currently recommended for multipin peptide synthesis is the milder Fmoc scheme, which is the only scheme described in this chapter.

Table 9.7.1 Types of Pins for Multipin Peptide Synthesis[a]

Name	Linker[b]	Physical format[c]	Loading	Final form of peptide
NCP	Noncleavable	Gear	50 nmol	(N-capping)-PEPTIDE-linker-pin
MPS	AA ester	Macrocrown	5 µmol	(N-capping)-PEPTIDE-acid
MPS	Rink amide	Macrocrown	5 µmol	(N-capping)-PEPTIDE-amide
DKP	DKP-forming	Gear	1 µmol	(N-capping)-PEPTIDE-DKP
GAP	Glycine ester	Gear	1 µmol	(N-capping)-PEPTIDE-glycine-acid

aAbbreviations: DKP, diketopiperazine; GAP, glycine acid peptide; MPS, multiple peptide synthesis; NCP, noncleavable peptide; (N-capping), a free amine, acetyl group, or biotin; PEPTIDE, the sequence of the peptide being made.

bNature of linker between peptide and graft polymer on the pin: noncleavable linker, β-alanine-hexamethylenediamine; DKP, diketopiperazine; AA ester, amino acid ester; Rink amide, Rink amide–forming linker.

cSee Figure 9.7.2B.

Before beginning to plan the actual synthesis in detail, a choice needs to be made regarding how the peptides will eventually be presented in the bioassay. The options available to investigators are listed in Table 9.7.1.

For noncleavable peptide (NCP) kits, peptides are permanently bound on the solid phase (pin surface) and can be used for direct binding assays but not for interaction with living cells or other complex (e.g., multicomponent) systems. In this case, the peptides must be "regenerated" between repeat assays by disrupting the peptide-ligand interaction without damaging the peptide. The quantity of peptide made is very small (50 nmol), but it is sufficient to provide a high surface density of peptide for direct binding assays.

In the other options, peptides are synthesized on pins and then released into solution. The mechanism of peptide release into solution affects the postsynthesis handling and thus the suitability of peptides produced by each cleavage method for various assay systems.

For multiple peptide synthesis (MPS) kits, the released peptides have a "native" free acid or an amide carboxy terminus. To make free acid C-termini, it is necessary to use macrocrowns that already have the first (C-terminal) amino acid on them because the chemistry of forming the first (ester) link is too difficult for the inexperienced user. In contrast, the Rink amide linker allows formation of a peptide with a C-terminal amide of any amino acid by adding the C-terminal amino acid to the Rink handle macrocrown using the standard amino acid coupling protocol. A Rink amide linker is a linker that can [illegible] an amino acid but then can be cleaved in trifluoroacetic acid (TFA) to release the amide form of that amino acid (Rink, 1987). Although acid or amine endings are often the most desirable peptide format to have, they are also the most complex to produce because the cleavage of the peptides from the pin is into neat TFA plus scavengers which needs to be evaporated to recover the peptide. The scale of peptide synthesis for MPS kits is 5 µmol (~5 mg of a decamer).

For glycine acid peptide (GAP) kits, peptides with a glycine at the carboxy terminus are cleaved as the free acid, so that the C-terminal residue is a natural amino acid (glycine) and is not blocked. The peptides are also relatively simple to release from the pin and require little postsynthesis handling. However, the presence of glycine at the C-terminus may be undesirable where the C-terminus plays an important role in peptide bioactivity. The scale of synthesis for GAP kits is 1 µmol (~1 mg of a decamer).

In diketopiperazine (DKP) kits, peptides are synthesized with a DKP group at the C terminus. The DKP group is a cyclic dipeptide formed from C-terminal lysine and proline residues during the facile cleavage of the peptide under the mildest possible conditions:

neutral aqueous buffer. In applications where the presence of the DKP group is acceptable, this type of peptide can make the downstream processing of synthetic peptides very simple and fast. The peptides can be placed into a bioassay system immediately after completing the cleavage. The scale of synthesis for DKP kits is 1 µmol (~1 mg of a decamer).

For these five kit options, it is also possible to choose a variety of N-terminal endings on the peptides. For example, it may be desirable to acetylate pin-bound peptides (see Support Protocol 2) to eliminate the positive charge that would otherwise be present on the α-amino group of the N-terminal residue, or to enhance the activity of a peptide in a T helper assay (Mutch et al., 1991). A handy option for cleaved peptides is to place a biotin group on the N-terminus (see Support Protocol 3) so the peptide can be captured using avidin or streptavidin. These additions must be made prior to side-chain deprotection of the peptides.

There are other configurations for multiple peptide synthesis—e.g., the SPOTS or "peptides on paper" system (Zenica/CRB), the RaMPS system (DuPont), and multi-synthesizer machines (e.g., Advanced ChemTech).

Assessing Peptide Sequences

Peptides differ so much in properties that it is important to assess the likely properties of the peptides before attempting to synthesize them. Peptide length and hydrophobicity are the two main attributes affecting successful synthesis. The longer the peptide, the lower will be the purity of the product, as each amino acid coupling cycle is never 100% efficient. Synthesis of peptides longer than 20 residues should be avoided unless special attention can be given to each sequence. Hydrophobic peptides may be difficult to synthesize, but more significantly they may be poorly soluble in aqueous buffers, restricting their ultimate usefulness in bioassays. Prior to beginning synthesis of a set of peptides, it is sensible to assess them all for hydrophobicity (Fauchere and Pliska, 1983; *UNIT 9.3*) and decide if all should be attempted as they stand. In many cases, it is possible to choose slightly different peptides (longer, shorter, or using a different starting and finishing point in the homologous protein sequence) that will have more user-friendly properties.

As well as these general factors affecting peptides, particular peptide sequences may have characteristics that make them difficult to synthesize, or they may be problematic after synthesis. It is not feasible to discuss all the common problems here. To help assessment of peptide sequences, a software application called Pinsoft is available free from Chiron Technologies. This allows any sequence to be typed in, and an assessment is automatically reported.

Generating Peptide Sequences

Computer software (Pepmaker) supplied with synthesis kits allows sets of overlapping peptide sequences to be generated from a protein sequence computer file using the single-letter amino acid code. Alternatively, sequences can be created using a word processor and the resulting computer text file can then be used by Pepmaker to guide synthesis. The use of this software simplifies the otherwise complex and tedious task of adding the right amino acids to each reaction plate on each synthesis cycle.

DO NOT expose pins to acetic anhydride at any other time except during acetylation. Also, do not store acetic anhydride anywhere near where peptide synthesis is performed.

The DMF does not need to be amine-free.

20% piperidine/DMF

Prepare a 20% (v/v) solution of the best quality piperidine available in analytical reagent–grade dimethylformamide (DMF). Prepare a fresh solution for each synthesis (solution can be reused several times within a synthesis). Store at room temperature in an amber bottle containing activated molecular sieves to remove moisture.

CAUTION: This solution is highly flammable and toxic.

If high-quality piperidine is not available, it may have to be treated with solid sodium hydroxide and redistilled.

DMF need not be amine-free.

Side chain deprotecting (SCD) solution

33 parts (v/v) trifluoroacetic acid
1 part (v/v) ethanedithiol
2 parts (v/v) anisole
2 parts (v/v) thioanisole
2 parts (v/v) H_2O
Prepare immediately before use and do not store or reuse

CAUTION: This solution is corrosive and extremely malodorous. Contamination of the laboratory, especially with ethanedithiol, should be avoided. Wipe the outside of ethanedithiol-contaminated equipment or containers with dilute, 0.1% aqueous hydrogen peroxide to oxidize ethanedithiol to a nonodorous compound before removing the container from the fume hood. DO NOT allow hydrogen peroxide to contact other readily oxidizable materials or reagents.

Sonication buffer

1% (w/v) SDS
0.1 M sodium phosphate buffer, pH 7.2
0.1% (v/v) 2-mercaptoethanol (2-ME)
Store at room temperature up to 1 week

CAUTION: Before discarding sonication buffer, destroy remaining 2-ME by adding 2 ml 30% hydrogen peroxide per liter of buffer.

COMMENTARY

Background Information

The multipin method was developed by Dr. H.M. Geysen and coworkers (Geysen et al., 1984, 1987) as a scanning method for linear antibody-defined epitopes. Eventually in the late 1980s, the method was adapted to parallel synthesis of cleaved (soluble) peptides (Maeji et al., 1990), opening the way for systematic scanning of T helper (Reece et al., 1993) and cytotoxic epitopes (Burrows et al., 1994). Initially only suitable for synthesis of short peptides (up to 10 amino acid residues), the method can now routinely produce peptides of up to 20 residues of acceptable quality for initial screening experiments (Valerio et al., 1993).

Critical Parameters

Successful peptide synthesis requires reagents of a quality appropriate to the particular step, and the careful application of those reagents. For example, the protected amino acids need to be free of reactive counterions such as dicyclohexylamine (DCHA), contaminating unprotected amino acid, isomers such as the D-amino acid, and water. Check carefully that the amino acid as supplied is EXACTLY the same as specified in the manual or on the software. Apart from quality testing each amino acid, the best assurance of quality is to buy only from reputable suppliers.

Dimethylformamide (DMF) is the primary solvent for carrying out reactions (couplings) on pins. Its low volatility and moderate polarity make it suitable for dissolving the amino acids and solvating the graft polymer/growing peptide on the pin surface. Purity is not critical for some (washing) steps, but is critical for the DMF used just before and during amino acid coupling. Presence of excessive amine in the DMF results in loss of activated amino acid because the amino acid couples to the amine rather than to the peptide on the pin. Fortunately, the pin system allows use of substantial molar excesses of incoming amino acid (typically 6- to 1000-fold), so loss of some amino acid is not disastrous. Fresh DMF of the best available grade should be used for the coupling, and it is recommended that the amine level be tested using the FDNB test (Stewart and Young, 1984).

Liberal use is made of methanol as a washing solvent. Analytical reagent grade methanol is readily available at low cost in large containers (20 or 200 liters) and is relatively easy to dispose of. It is possible to reduce the use of methanol by reusing it for washes: the last wash bath in any series should be in fresh (pure) methanol. In the next round of washes, the former last bath is then reassigned as the second-to-last wash, the previously second-to-last bath becomes the third-to-last, and so on. For each synthesis cycle, the first wash bath in the series is the one which is discarded. The presence of methanol is undesirable during reactions on the pins, but as it evaporates readily it can be easily removed by standing the block in a moving stream of air, such as the opening of an operating chemical fume hood. Methanol will dry more rapidly and the methanol-washed pins will take up less moisture from the air if the methanol is warm (e.g., prewarmed to 45°C in a closed bottle in a water bath).

Other solvents (e.g., ether, petroleum ether, acetonitrile) should be the best available grade.

Carrying out the correct synthesis of the peptides requires that all steps are performed with a very high level of attention to detail. All cyclically repeated steps (washes and deprotections) must be performed, and the activation and dispensing of the amino acids for each coupling cycle must be carried out exactly, or the peptides made may have the incorrect sequence, may be missing an amino acid, or may be truncated. Computerized equipment is available for assisting with the accurate dispensing of amino acids to the wells in a reaction tray (e.g., "Pin-Aid," Chiron Technologies; Carter et al., 1992). The growing peptides must not be subjected to conditions that would prematurely block or deprotect the side chains (for example, from premature exposure to acetic anhydride or trifluoroacetic acid which should be stored well away from where peptide synthesis is being performed).

As a spot test for correct completion of all the steps of synthesis, it is wise to synthesize controls on each block of 96 pins. For noncleavable peptides, these controls can be peptide sequences that can be probed with an antibody known to react with the peptide. In this case, one of the two peptides should be a negative control, such as a randomized sequence. For cleavable peptides, the quantity and quality of the controls can be monitored by the usual techniques of HPLC (*UNIT 9.2*), amino acid analysis, and mass spectrometry. Ultimately, proof that an assay result is a function of the particular peptide made has to rely on a confirmatory experiment carried out with more highly-characterized peptide or on analysis of a sample of the particular peptide used in the experiment.

Once peptides have been made, they need to be handled and stored carefully to prevent degradation. Noncleavable peptides (pins) should be stored dry in a refrigerator after removal of any bound protein. If stored with desiccant they should be stable for months to years. Cleaved peptides can be stored frozen or as dry powder. After a long period of storage, it is wise to reassay controls or confirm the quality of the stored peptide by analysis.

Another parameter critical to data from large numbers of peptides is to ensure that the identity of each peptide is properly tracked and that activity is not ascribed to the wrong peptide. Consistent use of the 8 × 12 microtiter plate format for synthesis, storage, assay, and use of computerized records for tracking all three processes can help avoid mistakes. Tracking and control is particularly easy if the assay data is read directly from a microtiter plate reader to a computer that is programmed with the peptide information because this method avoids manual data transcription.

Anticipated Results

For a noncleavable pin-peptide synthesis, two control peptides, one of which is reactive with a monoclonal antibody in ELISA and the other serving as a nonbinding peptide control, should show the specific binding expected based on past data. For cleaved peptides, the yield of control peptide should be in the range

expected from the stated pin loading (substitution level), e.g., 1 µmol for GAP and DKP kits or 5 µmol for the MPS kit. Purity of the cleaved controls should be consistent with the results of previous batches and should be of an acceptable standard.

Testing of a systematic set of peptides in a bioassay can give data that is interpretable without recourse to additional controls, because a systematic set of peptides through a protein includes many sequences that are unlikely to be reactive sequences, i.e., they act as internal negative controls. Figure 9.7.7 shows one set of ELISA data from scanning noncleaved peptides with a monoclonal antibody. In screening for T helper cell responsiveness it is critical to include many control cultures, not only controls with no peptide added but also controls

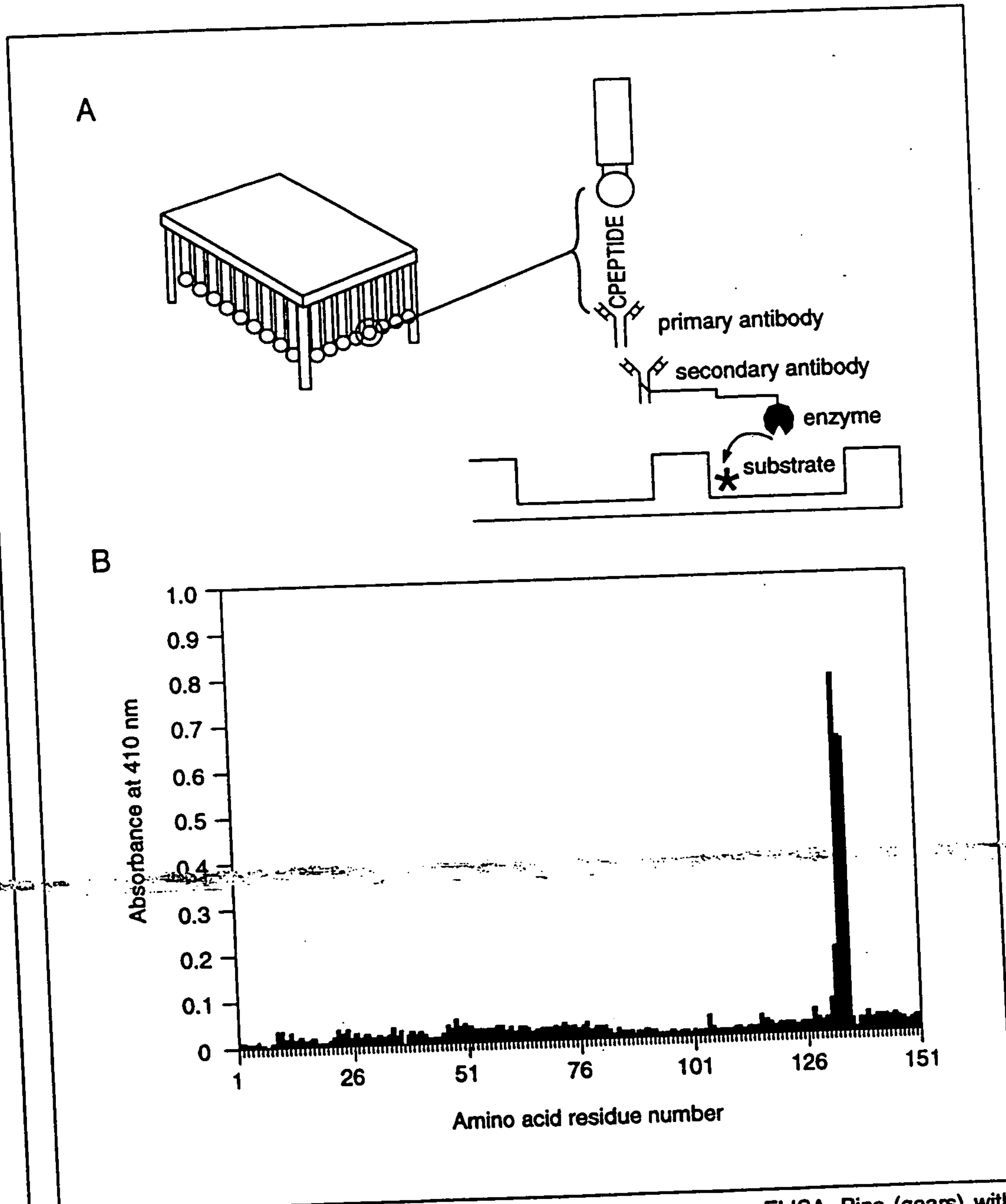

Figure 9.7.7 Multipin capture ELISA. **(A)** Setup for multipin capture ELISA. Pins (gears) with peptides covalently attached are incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance is measured and the resulting absorbance values are graphed versus peptide number, corresponding to the N-terminal residue number of the peptide in the protein sequence. **(B)** Peptide pin capture ELISA results with a monoclonal antibody against pins bearing octamer peptides of gonococcal pilin protein. All the peptides that show high readings contain a significant portion of the epitope. (Diagram courtesy of Dr. Fred Cassels, Walter Reed Army Institute of Research, Washington, D.C.)

with nonstimulatory peptide. Systematic sets of peptides automatically include such controls (Reece et al., 1994).

Time Considerations

If amino acid coupling is carried out at 3 cycles/day, which can fit into a conventional working day, then it will take up to 2 weeks to make a set of 15-mers, as there is extra time required for side chain deprotection and drying down (depending on the peptide format). Although this may seem slow, the fact that hundreds or thousands of peptides can be made simultaneously means that a project requiring large numbers of peptides is completed in a very short time. Indeed, the rate-limiting step may be the time it takes to carry out the assays on the large number of peptides when they become available.

From this perspective, biotinylated peptides produced on glycine acid peptide (GAP), diketopiperazine (DKP), or multiple peptide synthesis (MPS) pins have a great advantage over the noncleavable peptide (NCP) pin-bound peptides, as the latter can only be assayed once a day, whereas hundreds of parallel assays can be carried out on all biotinylated peptides at once. Reading data directly into a computer enables the massive amounts of data to be stored efficiently for later analysis.

Dispensing amino acids can be carried out efficiently by two people, one reading out the position into which the amino acid is to be dispensed and the other doing the actual dispensing. The passive partner (reader) can also act as a cross-checker to ensure no mistakes are made. If a computer-controlled pointing device is used, accuracy is improved and dispensing becomes a one-person operation. For large syntheses (>200 peptides), it is important that the dispensing be fast and accurate so that three couplings can be carried out per day.

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Journal of Immunological Methods 172 (1994) 241–254

JOURNAL OF IMMUNOLOGICAL METHODS

Scanning for T helper epitopes with human PBMC using pools of short synthetic peptides

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Received 9 November 1993; revised received 15 February 1994; accepted 8 March 1994

Abstract

Major T helper epitopes of medically important antigens can be located by measuring the proliferative responses of human peripheral blood mononuclear cells (PBMC) to pools of short synthetic peptides. The length and endings of the peptides used were shown to be critical for success in identifying Th cell epitopes. Many epitopes would be missed if either long (31mers) or short (less than 12mers) peptides were used. Pools of 14 and 16mers were more efficient than 12mers spanning the same region, however, for a promiscuous Th cell epitope of tetanus toxin (tt 947–967), two of three donors tested did not respond to 18mers or shorter peptides spanning this region. Although peptides with either unblocked or blocked ends were stimulatory, peptides with blocked ends were generally more efficient. The peptide concentration and number of available APC were also found to affect the efficiency of the proliferation assay as a measure of peptide recognition by Th cells.

Two screenings of the entire set of tetanus toxin peptide pools using different samples of PBMC from the same donor identified common major stimulatory regions. Thus, PBMC and peptide pools can be used for the reproducible identification of Th cell epitopes. After immunization with tetanus toxoid (TT), peptide-responsive cells increased in frequency in parallel to the increase in TT responsive cells, indicating that the peptide-responsive cells were primed by TT.

Key words: T helper cell; Epitope; Synthetic peptide; Peripheral blood mononuclear cell; Proliferation; Tetanus toxin

1. Introduction

Mapping of T helper cell epitopes within protein antigens has previously been achieved using the response of Th cell clones to protein fragments generated by various methods, including enzymic or chemical fragmentation (Demotz et al., 1989a,c), gene fragments generated by restriction enzyme digestion (Lamb et al., 1987) or PCR using synthetic oligonucleotide primers (Nakagawa et al., 1991). Synthetic peptides of 15–30 residues (Good et al., 1988; Ho et al., 1990; Brett et al., 1991) and short peptides synthesized using the multipin peptide system (Maeji et al., 1990; Gammon et al., 1990; Brown et al., 1991; Mutch

Abbreviations: TT, tetanus toxoid; tt, tetanus toxin; PBMC, peripheral blood mononuclear cells; β-dkp, β-amino-alanine-diketopiperazine; dkp, diketopiperazine.

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SSDI 0022-1759(94)00072-5

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et al., 1991; Suhrbier et al., 1991; Burrows et al., 1992; Rodda et al., 1993; Reece et al., 1993) have also been used.

Work with clonal Th cells has shown that the nature and length of the peptide containing the T cell epitope have important effects on its stimulatory ability. Peptides with blocked N- and C-terminal endings were demonstrated to be just as efficient or more efficient than unblocked peptides for activating T helper cells (Allen et al., 1989; Mutch et al., 1991; Gammon et al., 1991). In contrast, shorter peptides with unblocked endings containing the minimum epitope were shown to be more efficient for cytotoxic T cells (Bednarek et al., 1991).

TT processing and epitope formation by APC has been shown to vary for donors even with the same restriction element (Demotz et al., 1989b; Panina-Bordignon et al., 1989) and inbred mice of the same MHC haplotype (Gammon et al., 1990). This may be due to a requirement for specific protease(s) to generate particular epitopes. The use of shorter peptides, which require little or no processing to be active in Th cell assays (Mellins et al., 1990), avoids the requirement for specific proteases to generate epitopes. Peptides with as few as 8, 9 and 10 amino acids (plus the C-terminal tripeptide β-amino-alanine-diketopiperazine (β-dkp) moiety) have been shown to be stimulatory for Th cell clones (Suhrbier et al., 1991; Brown et al., 1991; Gammon et al., 1991). These lengths are consistent with peptides found bound naturally to class II antigens (Rudensky et al., 1991; Hunt et al., 1992).

Peripheral blood mononuclear cells (PBMC) have been used to map T helper cell epitopes using short synthetic peptides (Good et al., 1988; Brett et al., 1991; Russo et al., 1993; Reece et al., 1993). As only the $CD4^+$ subset of T cells is required for in vitro human T cell responses to conventional antigens such as TT (Via et al., 1990), this suggests that proliferation assays involving PBMC are measuring the activation of antigen-specific $CD4^+$ Th cells. PBMC are useful for mapping Th cell epitopes because they represent a repertoire not biased by prior in vitro selection of the best-growing or most frequent clones (Gammon et al., 1990).

A map of the Th cell epitopes of tt using human PBMC and pools of short synthetic peptides has been reported (Reece et al., 1993). The use of PBMC and the peptide pooling strategy has also been applied for mapping Th cell epitopes within other antigens such as influenza type A (two subtypes of HA, and NP) (Rodda et al., 1993); MPB-70 from *Mycobacterium bovis* (unpublished data), Lol pI (Bungy et al., 1993) and HIV antigens; gag and env (Mutch et al., unpublished observations).

This paper reports an assessment of the sensitivity and efficiency of the pooling/decoding method for identifying T helper cell epitopes using PBMC and pools of synthetic peptides. We have assessed the reproducibility of the method and the effect of peptide concentration and length of the peptides on the efficiency of Th cell recognition. Ways of optimizing recognition of Th cell epitopes, such as supplementing PBMC with APC, were examined.

We also addressed the question of whether PBMC responses to short peptides is due to the cross-reactivity of Th cells primed with a different antigen (Good et al., 1992). The specificity of proliferative responses of PBMC to tt peptide epitopes was tested by study of a donor before and after immunization with TT.

2. Materials and methods

To ensure that proliferation assays using PBMC were minimally affected by spontaneous proliferation, factors such as the culture medium, incubation time and labeling method were systematically investigated and optimized. All work was performed using the optimized method for the standard PBMC proliferation assay described below, except where specified.

2.1. Medium

'Incomplete' medium consisted of RPMI 1640 (CSL, Melbourne, Australia) supplemented with 2 mM L-glutamine, 5 mM Hepes buffer pH 7.4, and 20 μg/ml gentamicin. 'Complete' medium consisted of 10% (v/v) heat-inactivated human

serum, pooled from screened donations, or 10% (v/v) heat-inactivated autologous serum (obtained from defibrinated blood), added to 'incomplete' medium.

2.2. Antigens

Pin-made peptides were synthesized using the multipin peptide synthesis strategy (Maeji et al., 1990). Peptides had either blocked endings (i.e., an acetylated N-terminus and a C-terminal β-dkp or dkp group) or where noted, unblocked endings (free N- and C-terminal endings). Peptides were cleaved into sterile 0.1 M sodium bicarbonate or 0.1 M Hepes, pH 7.8, in sterile 96-well microtitre trays. The purity of representative peptides was assessed using HPLC and was generally found to be > 80%.

The following bulk peptides were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide synthesizer: P388 (H-QYIKANSKFIGITEL-OH, tt 830–844; Panina-Bordignon et al., 1989), P399 (Ac-QEIYMQHT-YPIS-β-dkp, tt 257–268), P442 (H-EQDPSGAT-TKSAMLTNLIIFGPGPVLNKNEV-OH, tt 141–171), P443 (H-SVDDALINSTKIYSYFPSVISKV-NQGAQGIL-OH, tt 581–611), P444 (H-DTQSK-NILMQYIKANSKFIGITELKKLESKI-OH, tt 821–851), P445 (H-IEYNDMFNNFTVSFWLR-VPKVSASHLEQYGT-OH, tt 941–971), P459 (Ac-VRDIIDDFTNESSQKT-NH_2, tt 616–631) and P480 (H-FNNFTVSFWLRVPKVSASHLE-OH, tt 947–967; Panina-Bordignon et al., 1989), P485 (Ac-IVKQGYEGNFIG-OH, tt 652–663), P486 (Ac-STIVPYIGPALN-OH, tt 640–651), [illegible]YSYFPSVISK-OH, tt 591–602). Peptides were purified to > 90% and their compositions were confirmed by amino acid analysis.

All peptides were screened for cytotoxic activity by co-culturing with PBMC and 10 μg/ml Con A (Sigma, St. Louis, U.S.A.).

TT was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia.

2.3. Cell preparations

Whole venous blood was drawn from volunteers who had given informed consent to venepuncture. PBMC were isolated from defibrinated or heparinized venous blood using Ficoll-Paque density centrifugation (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described by Bøyum (1968).

2.4. Standard PBMC proliferation assay

Peptide-stimulated proliferation assays using 2×10^5 PBMC per well were performed in 96-well round bottom microtitre plates (Nunc, Roskilde, Denmark). Antigens were added in 20 μl volumes to these microtitre plates followed by 180 μl of cells in complete medium to give a final volume of 200 μl per well. Because PBMC often exhibited a low frequency of T cells specific for individual Th epitopes, all assays were carried out using at least 16 replicates per test group. PBMC were incubated at 37°C in 5%CO_2 in humidified air. After 90 h proliferation was detected by pulsing with 0.25 μCi tritiated (methyl-3H) thymidine (40–60 Ci/mmol, Amersham Australia, Sydney) per well for 6 h. DNA was harvested onto glass fibre filter mats (Skatron, Sterling, VA, USA) and incorporated thymidine was measured in an LKB 1205 Betaplate liquid scintillation counter. All assays included at least 16 wells each of negative controls (20 μl of peptide cleavage buffer) and positive controls (TT at 1.0 or 0.1 Lf/ml in 20 μl cleavage buffer) plus 180 μl cell suspension.

2.5. Statistical methods

Large amounts of peptide-stimulated PBMC data clearly demonstrated that the cpm values [illegible] replicate cultures (wells) within each test group were not normally distributed (data not shown). This is a direct consequence of the random distribution of low numbers of peptide-specific Th cells among replicate wells and made it inappropriate to treat proliferation data (cpm) using statistical methods based on the Normal distribution. The Poisson model is a better model for the data (Taswell et al., 1984). To assign the results from individual wells as 'positive' or 'negative', a cutoff value of the mean plus three times the standard deviation of the cpm values for the

normally distributed unstimulated (Cells Alone) group was calculated (Taswell et al., 1984). Poisson statistics were then used to determine whether the difference in the frequency of positive wells between each test group and the Cells Alone group was significant. Where the data are significant at the 0.25% ($p < 0.0025$) or 5% ($p < 0.05$) level, results are reported as the frequency of positive wells in the test groups.

As this method is uncommon for analysis of proliferation tests, a typical set of data comparing this method of analysis with a conventional method using the mean ± SD of the ^{3}H-TdR uptake (cpm) has been reported (Reece et al., 1993). This comparison shows that where significant frequencies are reported, the mean of all replicates in a test group is higher than the mean of the Cells Alone control group, but the SD is also high, so a test based on normally distributed cpm data may not detect a difference between test groups and the Cells Alone controls. In contrast, if individual wells are scored as positive or negative, the statistical test based on a Poisson distribution can be used as a more sensitive and realistic way of looking for test groups significantly different from the Cells Alone control (Reece et al., 1993).

Precursor frequencies were estimated using the single-hit Poisson model (Taswell et al., 1984).

3. Results

3.1. Development of the method for T cell epitope mapping using PBMC

Proliferation of PBMC in response to incubation with sets of short synthetic peptides encompassing entire protein sequences was developed as a method for the detailed mapping of helper T cell determinants (materials and methods section). Dodecapeptides (12mers) with a constant 3-residue C-terminal extension (β-dkp) (Maeji et al., 1990) were employed for this work since previous studies have shown that peptides of this length are suitable for identifying epitopes using Th cell clones (Brown et al., 1991; Suhrbier et al., 1991; Gammon et al., 1991) and fall within the range of peptide lengths found binding naturally to MHC class II (Rudensky et al., 1991). As individual testing of every overlapping 12mer peptide of large proteins such as tt (1304 peptides) was impractical, a pooling/decoding strategy was devised. Using this strategy, the two most commonly recognized regions (Pools 30 and 42) corresponded to two published tt epitopes (Ho et al., 1989; Panina-Bordignon et al., 1989), showing that the pooling method is effective for identifying major Th cell epitopes (Reece et al., 1993).

To investigate whether the PBMC mapping method would give reproducible results with different aliquots of PBMC, one donor was scanned twice at an interval of 2 weeks (Table 1). This assay was performed using the standard proliferation assay (materials and methods section) except that a 138 h incubation period was used rather than 90 h. Results are reported as peptide pools scored positive at the $p < 0.05$ (+) and the $p < 0.0025$ (++) levels.

Table 1 shows that of the ten frequently stimulatory pools identified at the $p < 0.0025$ level in the first assay, seven were also frequently stimulatory ($p < 0.0025$) and two pools significantly stimulatory ($p < 0.05$) in the second assay. Only one pool (pool 12) identified at the $p < 0.0025$ level in the first assay was not stimulatory in the second assay. There were, however, five stimulatory pools (pools 18, 34, 45, 58 and 66) identified in the second assay at the $p < 0.0025$ level that were not identified in the first assay. The difference in the pools identified in the two scans may represent a temporal change in the dominance of epitopes (i.e., clones).

3.2. Comparison of the effectiveness of pools of overlapping 12mer peptides with single 31mer peptides spanning the same regions

Responses of several donors to each of three pools spanning three dominant epitope regions within the tt sequence (Reece et al., 1993) were compared to responses incurred using the single 31mer peptide containing all residues encompassed by that particular pool. A fourth peptide spanning a published 'promiscuous' T helper cell epitope (tt 947–967, Panina-Bordignon et al.,

Table 1

Complete scan of tetanus toxin for Th cell epitopes using two different PBMC samples from donor E [a]

Pool [b] no.	tt sequence spanned by pool	Donor E [c]	
		test 1	test 2
1	1– 31		
2	21– 51		
3 *	41– 69		
4	59– 89		
5	79– 109		+ [d]
6	99– 129		
7	119– 149		
8	139– 169	+ + [e]	+ +
9	159– 189		+
10	179– 209		+
11	199– 229		
12	219– 249	+ +	
13	239– 269		
14	259– 289		
15	279– 309	+	+
16	299– 329		
17	319– 349		+
18	339– 369		+ +
19	359– 389		
20	379– 409		
21	399– 429		
22	419– 449		
23	439– 469		
24	459– 489		
25	479– 509		
26	499– 529	+ +	+
27	519– 549		
28	539– 569	+ +	+ +
29	559– 589		
30	579– 609	+ +	+ +
31	599– 629		
32	619– 649	+ +	+ +
33	639– 669	+ +	+ +
34	659– 689		+ +
35	679– 709		
36	[illegible]		
37	719– 749		
38	739– 769	+	
39	759– 789		
40	779– 809		
41	799– 829		
42 *	819– 847	+ +	+ +
43	837– 867		
44	857– 887		
45	877– 907		+ +
46	897– 927		
47	917– 947		
48	937– 967		
49	957– 987		
50	977–1007		+
51	997–1027	+	
52	1017–1047		+
53	1037–1067		
54	1057–1087		
55	1077–1107		
56	1097–1127	+	+ +
57	1117–1147		
58	1137–1167		+ +
59	1157–1187	+	+ +
60	1177–1207	+ +	+ +
61	1197–1227		
62	1217–1247		
63	1237–1267		
64	1257–1287	+ +	+
65	1277–1307		
66 *	1297–1315		+ +
Total no. of positive pools [e]		10	14
Cells alone		1/112 [f]	1/112
TT (1.0 Lf/ml)		14/56 [f]	28/56

[a] A standard PBMC proliferation assay, with the exception of a 138 h incubation period, was used.

[b] Each peptide pool consisted of 20 overlapping 12mers (0.3 μM peptide/pool) unless specified by *.

[c] PBMC were from donor E taken at different times.

[d] Peptide pools scored positive at $p < 0.05$ using 16 replicates per test.

[e] Peptide pools scored positive at $p < 0.0025$ using 16 replicates per test.

[f] Number of positive wells over total number of wells used for these controls.

1989), found to be nonstimulatory using pools of 12mer peptides (Reece et al., 1993) was included to see if a single 31mer could detect an epitope where the pooling method had failed to do so.

The concentration of each peptide within the pool was 0.3 μM, whereas individual 31mers were tested over a range of concentrations (8.5,3.4,0.7 and 0.14 μM) to avoid bias against the long peptides due to the possibility of a suboptimal concentration being used. Results are represented as the number of positive wells out of 32 replicates. Only those data significantly different from the Cells Alone control ($p < 0.0025$) are shown (Table 2). To examine whether there was a need to include every possible overlapping 12mer in the pool for it to be stimulatory, pools were also tested as sets containing overlapping pep-

Table 2
Frequency of PBMC responses to pools of 12mer peptides compared to a single 31mer peptide spanning the same sequence as the pool [a]

12mer pool [b]	Donor	Test group						
		Pool [c]			31mer peptide (μM)			
		1 offset	2 offset	3 offset	8.5	3.4	0.7	0.14
	B	16 [d]	10	13	8	9	8	5
Pool 141–171	F	12	9	9	4	7	6	5
	I	24	24	18	21	16	22	21
	B	3	4	2	– [e]	–	–	–
Pool 581–611	F	3	3	–	–	–	–	–
	H	12	3	–	–	–	11	–
	I	8	13	12	–	–	6	3
	F	8	5	4	–	–	–	–
Pool 821–851	H	12	10	10	–	–	–	3
	I	16	20	11	2	13	4	3
	B	–	–	–	–	–	4	–
Pool 941–971	F	–	–	–	–	–	–	–
	H	–	–	–	–	–	–	–

[a] A standard PBMC proliferation assay (materials and methods section) with a 90 h incubation period was employed.
[b] Pool 141–171 corresponds to 31mer P442; pool 581–611 corresponds to 31mer peptide P443; pool 821–851 corresponds to 31mer peptide P444; pool 941–971 corresponds to 31mer peptide P445.
[c] Each pool contained either twenty; ten or seven overlapping 12mer peptides depending on whether the offset was 1, 2 or 3. Individual peptides within each pool were at a concentration of 0.3 μM.
[d] Number of positive wells in test groups significantly different from the Cells Alone control ($p < 0.0025$) using 32 replicates per test.
[e] Indicates not significantly different from Cells Alone ($p > 0.0025$).

tides offset by 1, 2 or 3 residues in their 'start' or N-terminal amino acid.

As expected, peptide pools spanning tt 581–611 and tt 821–851 induced proliferation of PBMC. However, the corresponding 31mer peptides gave little or no stimulation at any peptide concentration tested (Table 2). In contrast, the single peptide P442, spanning tt 141–171, was stimulatory but still tended to give a lower frequency of positives than the corresponding pool. Table 2

Table 3
The effect of peptide length and offset on the frequency of PBMC responses to pools of peptides spanning tt 587–609 [a]

Donor	Peptide offset [b]	Peptide length [c]			
		10mer	12mer	14mer	16mer
B	1	6 [d]	18	23	23
	2	8	14	21	23
	3	–	–	17	14
D	1	–	6	10	14
	2	–	–	9	12
	3	–	5	10	8

[a] A standard PBMC proliferation assay (materials and methods section) with a 90 h incubation period was employed.
[b] Increment in N-terminal residue number of consecutive overlapping peptides in the pool.
[c] Each peptide was tested at a concentration of 1 μM.
[d] No. of positive wells out of 32 replicate wells. Only frequencies of positive wells that were significantly different from the Cells Alone control are shown ($p < 0.0025$). – denotes not significantly different from Cells Alone ($p > 0.0025$).

also shows that as the residue offset increases, the frequency of positive responses tends to decrease.

All donors tested were unresponsive to pool 941–971 and the corresponding 31mer peptide P445, which contains the known T cell epitope tt 947–967 (Panina-Bordignon et al., 1989). As these donors were able to respond to the exact published T cell epitope (Table 4 and data not shown), this suggests that detection of some epitopes is dependent on the choice of 'correct' peptide length (see below).

3.3. *The effect of peptide length in scanning with pooled peptides*

To investigate the effect of peptide length on the efficiency of detection of Th cell epitopes using PBMC, four sets of overlapping peptides of lengths; 10, 12, 14 and 16 residues (plus the

Table 4
A comparison between the 21mer bulk peptide, P480, tt 947–967 and pools of 12, 15, 18 and 21mer peptides spanning tt 946–968 [a]

Peptide test/ length	Sequence	Donors D	Donors F	Donors I
Pool of 12mers	MFNNFTVSFWLR FNNFTVSFWLRV NNFTVSFWLRVP NFTVSFWLRVPK FTVSFWLRVPKS TVSFWLRVPKSA VSFWLRVPKSAS SFWLRVPKSASH FWLRVPKSASHL WLRVPKSASHLE LRVPKSASHLEQ	+ [b]	– [c]	–
Pool of 15mers	MFNNFTVSFWLRVPK FNNFTVSFWLRVPKV NNFTVSFWLRVPKVS NFTVSFWLRVPKVSA FTVSFWLRVPKVSAS TVSFWLRVPKVSASH VSFWLRVPKVSASHL SFWLRVPKVSASHLE FWLRVPKVSASHLEQ	+	–	–
Pool of 18mers	MFNNFTVSFWLRVPKVSA FNNFTVSFWLRVPKVSAS NNFTVSFWLRVPKVSASH NFTVSFWLRVPKVSASHL FTVSFWLRVPKVSASHLE TVSFWLRVPKVSASHLEQ	+	–	–
Pool of 21mers	MFNNFTVSFWLRVPKVSASHL FNNFTVSFWLRVPKVSASHLE NNFTVSFWLRVPKVSASHLEQ	+	+	+
P480	FNNFTVSFWLRVPKVSASHLE	+	+	+

[a] A standard proliferation assay (materials and methods section) with a 90 h incubation period was employed.
[b] Individual peptides within pools and P480 were tested at 4, 1 and 0.25 μM using 24 replicates per test. Positives (+) were scored if the number of responding wells was significantly different from the Cells Alone control at the $p < 0.0025$ level for any of the concentrations tested.
[c] – denotes not significantly different from the Cells alone control ($p > 0.0025$).

β-dkp tripeptide moiety) spanning the region tt 587–609 (Ho et al., 1990), were synthesized. All peptides of the same length were pooled (1 μM peptide/pool) and tested for their ability to stimulate PBMC from donors B or D. For each peptide length, three pools of peptides were tested, stepping along the sequence with an offset of 1, 2 or 3 residues (Table 3). Results are reported in the same way as in Table 2.

Table 3 shows that as the length of the peptides increases, the frequency of positive responses also increases. In particular, note that 10mer peptides were unable to stimulate PBMC from donor D whereas 14 and 16mer peptides were very effective. As peptide offset increases, the frequency of positive responses decreases, and proliferative responses to pools of shorter peptides (10 and 12mers) offset by 2 or 3 may not be seen. This confirms observations in Table 2 and suggests these pools may not contain the stimulatory sequence, which would be expected if both the length and 'frame' of the epitope within the peptide were important.

For 14mer and 16mer peptides, pools containing peptides offset by 2 were just as effective as those offset by 1, and significant responses were also obtained using 14mer and 16mer peptides offset by 3. These results suggest that a sequence can be successfully scanned with a subset of all possible overlapping peptides (of a given length) spanning the sequence.

3.4. Detailed investigation of the tetanus toxin region tt 947–967 containing a promiscuous epitope

None of the donors tested using the pools of 12mer peptides responded to the region covering promiscuous T cell epitope tt 947–967 (Panina-Bordignon et al., 1989; Reece et al., 1993). Table 2 also shows that the 31mer peptide, P445 (tt 941–971), was not stimulatory for donors B, F and H. In contrast, donors B, D, F, H and I were found to respond to P480, the 21mer peptide corresponding to the published sequence tt 947–967 (Table 4; data not shown for B and H).

To see whether the lack of stimulation by

Table 5
The effect of peptide concentration and unblocked versus blocked endings on the effectiveness of Th cell recognition [a]

Peptide concentration (μM)	Peptide			
	tt 257–268 (donor I)		tt 591–602 (donor E)	
	Blocked endings [b]	Unblocked endings [c]	Blocked endings	Unblocked endings
16	10 [d]	–	7	10
8	–	5	3	18
4	6	–	4	9
2	4	–	3	14
1	–	–	4	10
0.5	–	–	–	8
0.25	–	–	4	5
0.125	–	–	–	–
0.0625	–	–	–	–
0.031	–	–	–	–
0.016	–	–	–	–
0.0078	–	–	–	–

[a] A standard PBMC proliferation assay with a 90 h incubation period was employed.
[b] 12mer peptides with an acetylated amino terminus and a dkp moiety at the carboxy terminus.
[c] 12mer peptides with free carboxy and amino termini.
[d] no. of positive wells out of 32 replicate wells. Only frequencies that were significantly higher than the Cells Alone control ($p < 0.0025$) are shown.
– indicates not significantly different from Cells Alone ($p > 0.0025$).

shorter peptides was due to a need for the stimulatory sequence to consist of more than 12 antigen-homologous residues, four sets of peptides (12, 15, 18 and 21mers) spanning tt 946–968 were tested for stimulation. Peptides of the same length were pooled together (Table 4) and tested in parallel with P480 at three concentrations; 4, 1 and 0.25 μM. Data in Table 4 is reported as positive (+) if the frequency of positive wells was significant ($p < 0.0025$) at any of the concentrations tested.

Of the three donors tested, only donor D responded to pools of all peptide lengths tested (Table 4). In contrast, donors F and I responded to the pool of 21mer peptides and P480, but did not respond to peptides shorter than 21 residues. This indicates that the Th cell epitope for these two donors must be longer than 18 residues for it to be stimulatory.

3.5. The effect of peptide concentration and unblocked versus blocked endings on the effectiveness of T helper cell epitope mapping

Previous reports have shown that peptides with different endings differ in their ability to stimulate Th cell clones (Allen et al., 1989; Mutch et al., 1991; Gammon et al., 1991). The effect of peptide concentration was examined by testing two donors (I and E) against two 12mer peptides with blocked or unblocked endings. Peptides were tested at a range of doubling dilutions from 16 μM to 0.0078 μM. Table 5 shows that the concentration of peptide required to stimulate Th

Table 6
A comparison of the efficiency of individual blocked and unblocked peptides for the identification of a Th cell epitope within the A/Bangkok/1/79 haemagglutinin sequence 425–470 [a]

A/Bangkok [b] start residue	Sequence	Donor [c]					
		Flu1			Flu2		
		Blocked [d] 13mers	Unblocked [e] 13mers	Unblocked 16mers	Blocked 13mers	Unblocked 13mers	Unblocked 16mers
425	LEKYVEDTKIDLW(SYN)	–	–	–	–	–	–
427	KYVEDTKIDLWSY(NAE)	7 [f]	–	–	–	–	–
429	VEDTKIDLWSYNA(ELL)	–	–	14	–	–	–
431	DTKIDLWSYNAEL(LVA)	16	8	13	–	–	–
433	KIDLWSYNAELLV(ALE)	17	10	13	–	–	–
435	DLWSYNAELLVAL(ENQ)	17	12	15	7	–	–
537	WSYNAELLVALEN(QHT)	7	–	–	4	–	6
439	YNAELLVALENQH(TID)	–	–	–	14	–	5
441	AELLVA[illegible]	–	[illegible]	[illegible]	7	[illegible]	5
443	LLVALENQHTIDL(TDS)	–	–	–	7	–	–
445	VALENQHTIDLTD(SEM)	–	–	–	–	–	–
447	LENQHTIDLTDSS(MNK)	–	–	–	–	–	–
449	NQHTIDLTDSEMN(KLF)	–	–	–	–	–	–
451	HTIDLTDSEMNKL(FEK)	–	–	–	–	–	–
453	IDLTDSEMNKLFE(KTR)	–	–	–	–	–	–
455	LTDSEMNKLFEKT(RRQ)	–	–	–	–	–	–

[a] A standard PBMC proliferation assay with a 90 h incubation period was employed.
[b] Start residue of peptides spanning A/Bangkok/1/79 haemagglutinin sequence 425–470. Individual peptides were tested at a concentration of 3 μM.
[c] Flu1 and Flu2 refer to two donors known to respond to a Th cell epitope within A/Bangkok/1/79 425–461.
[d] Blocked refers to acetylated peptides with dkp endings.
[e] Unblocked refers to non-acetylated peptides with free acid endings.
[f] no. of positive wells out of 24 replicate wells. Only frequencies that were significantly higher than the Cells Alone control ($p < 0.0025$) are shown. – denotes not significantly different from Cells Alone ($p > 0.0025$).

Table 7
The effect of the addition of APC to P459- and TT-stimulated PBMC on tritiated thymidine incorporation and frequency of positives [a]

Cells used	Cells alone		P459		TT
	Mean cpm (SD)	Cutoff [b]	10 μM	1 μM	0.5 Lf/ml
PBMC	288 (96)	576	33/48 [991 ± 381] [c]	21/48 [870 ± 332]	48/48 [9651 ± 2726]
PBMC + APC [d]	275 (75)	499	46/48 [1220 ± 590]	45/48 [797 ± 262]	48/48 [8074 ± 1526]

[a] A standard PBMC proliferation assay with a 90 h incubation period was employed.
[b] The cutoff cpm value was calculated as the mean plus 3SD for all the wells in the Cells Alone group ($n = 48$).
[c] The frequency of positive wells out of 48 replicates and mean cpm ± SD of these positively responding wells. All six test groups were significantly different from the Cells Alone ($p < 0.0025$).
[d] APC = 100000 irradiated (3000 Rads) autologous PBMC per well.

cells depends on the donor and peptide sequence tested. For donor I, > 1 μM peptide was required for stimulation whereas for donor E, stimulation was seen around 0.25 μM. Peptides with blocked endings were more effective with the 12mer peptide tt 257–268 (donor I) whereas the unblocked 12mer 591–602 (donor E) was more effective.

The effect of peptide endings was further examined with a set of overlapping 13mer peptides with either unblocked or blocked endings, and a set of 16mer peptides with unblocked endings, spanning a stimulatory region of the influenza strain A/Bangkok/1/79 hemagglutinin sequence (Benstead et al., in preparation). The individual peptides were tested at 3 μM against two donors and results are presented as the number of positive wells out of 24 replicates for test groups differing from the Cells Alone controls ($p <$ 0.0025) (Table 6).

For both donors, the frequency of positive responses to acetylated 13mer peptides with a β-dkp ending was higher than for peptides with free endings (Table 6). However, the 16mer peptides with free endings were of similar effectiveness to 13mer peptides with blocked endings. This indicates that longer peptides are more effective, so if peptides with free endings are used it would be advisable to use peptides of about 16 residues length.

3.6. *The effect of the addition of APC on the frequency of positive responses of PBMC to peptide*

Orosz et al. (1987) showed that for optimal proliferation when using low numbers of PBMC/well, supplementation with additional APC was necessary. Consequently, we investigated the effect of adding APC (irradiated autologous PBMC) to 2×10^5 PBMC/well to see if the availability of APC was a limiting factor.

PBMC from donor I, known to have a low Th precursor frequency to the tt peptide P459 (data

Table 8
Estimations of precursor frequencies/100000 PBMC, before and after immunization with TT: donor CM16 [a]

Test group	Peptide (1 μM)								TT (Lf/ml)		NP (μg/ml)
	P388	P399	P442	P459	P480	P485	P486	P487	1.0	0.1	110
Before [b]	0.2 [d]	0.9 *[e]	1.0 *	0.1	0.2	0.3	1.8 *	0.39	9.49 *	1.97 *	9.08 *
After [c]	6.3 *	13.1 *	18.5 *	0.8	1.3	6.9 *	24.6 *	1.74	> 77.43 *	76.58 *	9.81 *

[a] A standard PBMC proliferation assay with a 90h incubation period was employed.
[b] PBMC isolated just prior to immunization with TT.
[c] PBMC isolated 3 weeks after TT immunization.
[d] Precursor frequency estimations of the no. of peptide and antigen specific Th cells/100000 PBMC.
[e] * denotes significantly different from Cells Alone ($p < 0.0025$). More replicates were used in the Cells Alone group in the before group, giving these precursor frequency estimations a higher precision.

not shown), were tested with and without the addition of 1×10^5 irradiated PBMC (3000 Rads) (Table 7). The addition of APC increased the frequency of positive responses to tetanus toxin epitope P459 (tt 616–631) at 10 μM and 1 μM without significantly altering the magnitude of thymidine incorporation (Table 7). This indicates that the number of APC is limiting even when using 2×10^5 PBMC per well, and suggests that to detect all specific Th cells, extra APC need to be added to PBMC.

3.7. Are proliferative responses to tt peptides a result of Th cells responding to cross reactive antigens?

To confirm that PBMC responses to tt-homologous peptides are due to Th cells primed by TT rather than by cross reactive antigens, we examined proliferative responses of PBMC to a series of common tt Th cell epitopes before and after immunization of a volunteer with a TT booster (Table 8).

Significant increases in the precursor frequency estimations of Th cells specific for peptides P388, P399, P442, P485, P486 and TT were found 3 weeks after the TT immunization. In contrast, the frequencies of Th cells to two control antigens, influenza nucleoprotein (NP) and PPD (data not shown) did not alter after the TT immunization.

4. Discussion

We sought to combine the use of short synthetic peptides, which require little or no processing to be active in T helper cell assays (Mellins et al., 1990), with the use of PBMC as a source of polyclonal T cells. Although the mapping of Th cell epitopes using PBMC provides a repertoire not biased by the in vitro selection of the best-growing or most frequent clones (Gammon et al., 1990), working with PBMC poses difficulties not found when working with Th cell clones.

One major difficulty working with PBMC is the occurrence of sporadic (non-antigen-specific) proliferation in unstimulated PBMC cultures. The occurrence of 'false' positives is increased when culture conditions are not optimized, so factors such as media components must be screened to ensure the lowest background stimulation, while still supporting strong antigen-driven proliferation. A second difficulty arises due to the relatively low frequencies of Th cell precursors specific for a particular epitope. A large number of cells must therefore be used to ensure that significant numbers of peptide-specific Th cells are present. These can be distributed into a large number of replicate wells per test group to permit a statistical test of the difference between the control (Cells Alone) and each test group to be made. Based on theoretical considerations and our observations, we determined that conventional methods of data analysis (S.I., mean $\pm$ SD, net cpm etc.) were inappropriate, so an algorithm was developed which calculates a cutoff cpm value to enable those wells exhibiting significant proliferative responses to be objectively scored. A statistical test using Poisson statistics then differentiates positive tests groups from negative test groups and the Cells Alone controls (Geysen et al., in preparation).

A further restriction when using short synthetic peptides is the large number of peptides required to scan a whole protein sequence. This restriction can be overcome by synthesizing peptides using multipin peptide synthesis systems (Bray et al., 1990; Maeji et al., 1990). Testing overlapping peptides as pools reduces the limitation created by the limited availability of PBMC. The peptide pooling strategy was found to be an effective and simple method for identifying immunodominant Th regions within a protein (Bungy et al., 1993; Rodda et al., 1993; Reece et al., 1993).

To assess the effectiveness of the peptide pooling strategy compared to a conventional method using longer synthetic peptides (Good et al., 1988; Ho et al., 1990; Brett et al., 1991), pools of 12mer peptides were compared with 31mers spanning the same sequence. These results showed that the pools of short peptides were more efficient than longer peptides (Table 2). There may be a block in the recognition of longer synthetic peptides by helper T cells due to poor uptake from the medium or a requirement for specific protease(s)

to generate particular epitopes. Antigen processing and epitope formation by APC has been shown to vary even for donors with the same restriction element (Panina-Bordignon et al., 1989) and inbred mice of identical MHC haplotype (Gammon et al., 1990).

The most efficient length of peptide for specific stimulation of PBMC is unknown. Factors such as uptake by MHC class II, and whether or not processing of the peptide is necessary, affect the efficiency of epitope detection using polyclonal T cells (PBMC). Although pools of 12mer peptides were shown to be more effective than 31mers, we found that slightly longer peptides (up to 16 residues) were even more efficient for identifying Th cell epitopes (Table 3). Peptides in the 13–18 residue range have been found by extraction from purified class II molecules (Rudensky et al., 1991; Hunt et al., 1992) which suggests that synthetic peptides similar in length to the native peptides are the most efficient for detecting T cell epitopes.

In one instance short peptides were not successful in identifying a major Th cell epitope. Donors did not respond to pools of 12mer peptides spanning the promiscuous Th cell epitope tt 947–967 (Panina-Bordignon et al., 1989) even though they responded to a peptide corresponding to the published 21mer sequence (Reece et al., 1993). Further screening of 12, 15, 18 and 21mer peptide pools showed that of the three donors tested, only one responded to all peptide lengths while the other two responded only to 21mer peptides (Table 4). Thus, for some donor/epitope combinations, the peptide must be greater than 18 residues long to be stimulatory.

Overall these results show that some Th cell epitopes may be missed if peptides are too long (31 residues) or too short (12 residues). Therefore a peptide length between 16 and 21 residues is probably suitable for the identification of the majority of Th cell epitopes. Pools containing 16mer peptides offset by two residues can be just as efficient as pools of peptides offset by 1 (Tables 2 and 3), halving the number of peptides otherwise required for complete scanning.

Because short peptides require little or no processing to be active in Th cell assays (Mellins et al., 1990), some peptide responses may occur as a result of activating Th cells primed with a cross-reactive antigen (Good et al., 1992). To investigate if responses to tt peptides were due to priming with TT, we examined changes in the frequencies of Th cells to TT, or to tt-homologous peptides, as a result of immunization with TT (Table 8). The frequencies of Th cells to five tt peptide epitopes increased in parallel with increased frequencies to TT, indicating that PBMC responses to tt peptides are not due to Th cells generated to fortuitously cross reactive antigens. A specificity test (Hensen and Elferink, 1984) of PBMC responses also showed specificity of peptide-reactive PBMC for TT, and vice versa (data not shown).

We found that peptides with blocked endings were as efficient or more efficient for activating Th cells than unblocked peptides (Tables 5 and 6). This confirmed previous observations using Th cell clones (Allen et al., 1989; Mutch et al., 1991; Gammon et al., 1991) and may be due to the resistance of peptides with blocked endings to proteolytic action. These results contrast with cytotoxic T cells where shorter peptides with unblocked endings containing the minimum epitope were found to be more efficient (Bednarek et al., 1991).

The concentration of peptide required to stimulate Th cells depended on the peptide and donor tested. For one donor-peptide system, > 2 μM peptide was required for stimulation whereas another peptide-donor system required only 0.25 μM peptide. This information implies that some Th cell epitopes may not have been detected using 0.3 μM of each peptide in peptide pools (Reece et al., 1993), even though the effective concentration can be two or three times this level due to epitope sharing between overlapping peptides. Collawn et al. (1989) also showed that although most Th clones respond to 0.3 μM peptide, the optimum concentration can be higher or lower.

Although the frequencies of Th cells for many other antigens are lower than for TT (Van Oers et al., 1987), we have found this method for epitope scanning with PBMC can still be applied

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(Bungy et al., 1993). In summary, the distinctive features of the method as described include (i) the medium, especially the use of autologous serum; (ii) the incubation conditions, especially the use of round-bottom wells and a short incubation (90 h); (iii) data analysis which recognizes the spontaneous occurrence of positives in control and test groups; (iv) the use of large numbers of replicates to allow proper statistical evaluation of data; (v) pooling of short peptides; (vi) the choice of peptide length in the 14–21 residue range, a size not requiring further processing for presentation; and (vii) the use of shorter peptides which are active when their N- and C-terminal ends are blocked.

Acknowledgments

We thank Megan Jay, the Pin Peptide Synthesis team, and the generous blood donors for their help.

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Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3998–4002, July 1984
Biochemistry

Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid

(antigenic determinant/foot-and-mouth disease virus)

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Communicated by G. J. V. Nossal, March 12, 1984

ABSTRACT A procedure is described for rapid concurrent synthesis on solid supports of hundreds of peptides, of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner an immunogenic epitope of the immunologically important coat protein of foot-and-mouth disease virus (type O_1) is located with a resolution of seven amino acids, corresponding to amino acids 146–152 of that protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope was synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. It was found that the leucine residues at positions 148 and 151 were essential for reaction with antisera raised against intact virus. A lesser contribution was derived from the glutamine and alanine residues at positions 149 and 152, respectively. Aside from the practical significance for locating and examining epitopes at high resolution, these findings may lead to better understanding of the basis of antigen–antibody interaction and antibody specificity.

Recombinant DNA technology now makes possible by deduction from the determined nucleotide sequences reliable amino acid sequences of biologically important proteins. However, methods for identifying the loci in a protein that constitute the antigenic and immunogenic epitopes are few and time consuming and form the bottleneck to further rapid progress. Immunogenic epitopes are defined as those parts of a protein that elicit the antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule (1–3). On the other hand, a region of a protein molecule to which an antibody can bind is defined as an antigenic epitope. Antisera prepared against chemically synthesized peptides corresponding to short linear tracts of the total polypeptide sequence have been shown to react well with the native protein (4–9). However, interactions were also found to occur even when the site of interaction did not correspond to an immunogenic epitope of the native protein. This has been interpreted to mean that the number of immunogenic epitopes of a protein is less than the number of antigenic epitopes (4). Conversely, since antibodies produced against the native protein are, by definition, directed to the immunogenic epitopes, it follows that peptides reacting with these antibodies must contain elements of the epitopes. From a study of the few proteins for which the determinants have been accurately mapped, it is postulated that a determinant may consist of a single element (continuous) or of more than one element brought together from linearly distant regions of the polypeptide chain by the folding of that chain as it exists in the native state (discontinuous) (10). Systematic mapping of all the detectable reactive elements of a protein by the chemical synthesis of overlapping segments has until now been severely limited by the scale of the synthetic and testing capability required (10, 11). Smith and co-workers (12, 13) circumvented the decoupling and purification steps by combining solid-phase peptide synthesis and solid-phase radioimmunoassay using the same solid support.

We describe here the concurrent synthesis of all 208 possible overlapping hexapeptides covering the total 213-amino acid sequence of the immunologically important coat protein (VP1) of foot-and-mouth disease virus (FMDV), type O_1 (Fig. 1). The peptides, still attached to the support used for their synthesis, were tested for antigenicity by an ELISA using a variety of antisera. After identification of a hexapeptide reactive with antibody raised against the intact virus, all 120 hexapeptides representing the complete single point amino acid replacement set were synthesized and tested for retention of antigenicity. By this method a whole virus epitope was examined at a resolution of a single amino acid.

MATERIALS AND METHODS

Synthesis of Peptides. Polyethylene rods (diameter, 4 mm; length, 40 mm) immersed in a 6% (vol/vol) aqueous solution of acrylic acid were γ irradiated at a dose of 1,000,000 rads (1 rad = 0.01 gray) (15). Rods so prepared were assembled into a polyethylene holder with the format and spacing of a microtiter plate. Subsequent reactions at the tips of the rods were carried out in a Teflon tray with a matrix of wells to match the rod spacing. Conventional methods of solid-phase peptide chemistry (16, 17) were used to couple N^{α}-*t*-butyloxycarbonyl-L-lysine methyl ester to the polyethylene/polyacrylic acid via the N^{ϵ}-amino group of the side chain. Carboxy substitution of the support was determined by treating NH_2-lysine(OMe)-polyethylene/polyacrylic acid with ^{14}C-labeled butyric acid and was found to be 0.15–0.2 nmol/mm^2. Removal of the *t*-butyloxycarbonyl group was followed by the coupling of *t*-butyloxycarbonyl-L-alanine to complete a peptide-like spacer. Successive amino acids were added as dictated by the sequence to be synthesized. At the completion of the final coupling reaction, and after removal of the *t*-butyloxycarbonyl protecting group, the terminal amino group was acetylated with acetic anhydride in dimethylformamide/triethylamine. All *N,N*-dicyclohexylcarbodiimide-mediated coupling reactions were carried out in dimethylformamide in the presence of *N*-hydroxybenzotriazole. The following side-chain protecting groups were used: *O*-benzyl for threonine, serine, aspartic acid, glutamic acid, and tyrosine; carbobenzoxy for lysine; tosyl for arginine; 4-methylbenzyl for cysteine; and 1-benzyloxycarbonylamido-2,2,2-trifluoroethyl for histidine. Side-chain-protecting groups were removed by treatment with borontris(trifluor-

Abbreviations: FMDV, foot-and-mouth disease virus; P_i/NaCl, phosphate-buffered saline.

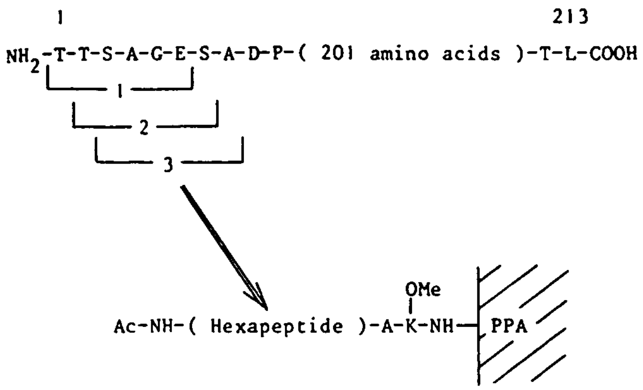

FIG. 1. The 213-amino acid sequence of VP1 (FMDV, type O_1) as translated by Kurz *et al.* (14) was subdivided into hexapeptide units, and each was synthesized on a separate polyethylene support in the orientation, and with a dipeptide spacer, as shown. Peptides are numbered according to the position of the NH-terminal amino acid within the VP1 sequence. PPA, polyethylene/polyacrylic acid.

acetate) in trifluoroacetic acid for 90 min at room temperature (18). After hydrolysis with HCl/propionic acid, sequences included in the synthesis as controls were analyzed to confirm that, although coupling at each stage had occurred, it was incomplete for several of the amino acids, notably arginine. Before testing by ELISA, support-coupled peptides were washed several times with phosphate-buffered saline (P_i/NaCl).

Antisera. Antisera against the intact virus particle were prepared by immunizing rabbits with 50 μg of inactivated, density gradient-purified virus in complete Freund's adjuvant. The animals were bled 3–4 weeks after the single inoculation. Anti-virus-subunit serum was prepared by inoculating rabbits three times, 3–4 weeks apart, with 10 μg of acid-disrupted purified virus, initially in complete Freund's and subsequently in incomplete Freund's adjuvant. The polypeptide VP1 was separated from the mixture of proteins obtained from urea-disrupted purified virus by isoelectric focusing (19). It was eluted from the gel with 8 M urea and dialyzed against P_i/NaCl, and antiserum was raised in rabbits as described for the virus subunit. Antiserum for scan 3 (see Fig. 2) was that used for scan 2 after absorption with purified virus (1500 μg of whole virus was incubated with 1 ml of serum for 72 hr at 4°C), and all virus-bound antibodies were removed by centrifugation.

ELISA. Support-coupled peptides were precoated with 10% horse serum/10% ovalbumin/1% Tween 80 in P_i/NaCl for 1 hr at 37°C to block nonspecific absorption of antibodies. Overnight incubation at 4°C in a 1:40 dilution of antiserum in the preincubation mixture was followed by three washes in 0.05% Tween 80/P_i/NaCl. Reaction for 1 hr at 37°C with a 1:50,000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase in the preincubation mixture was again followed by extensive washing with P_i/NaCl/Tween to remove excess conjugate. The presence of antibody was detected by reaction for 45 min with a freshly prepared developing solution (40 mg of *o*-phenylenediamine and 20 μl of hydrogen peroxide in 100 ml of phosphate buffer, pH 5.0), and the color produced was read in a Titertek Multiskan (Flow Laboratories, Melbourne, Australia) at 420 nm. Prior to retesting, bound antibody was removed from the peptides by washing peptides three times at 37°C in 8 M urea/0.1% 2-mercaptoethanol/0.1% sodium dodecyl sulfate and then several times with P_i/NaCl.

RESULTS

Identification of a Virus Particle-Associated Immunogenic Epitope. All 208 possible hexapeptides from the amino acid sequence of the VP1 protein of FMDV type O_1 were synthesized in duplicate. The amino acid sequence had been deduced from the nucleotide sequence of the VP1 gene (14). The results obtained for all the synthesized hexapeptides when tested by ELISA with six different antisera are shown in Fig. 2. Antisera used in the test were as follows: two different anti-(intact virus, type O_1), a virus-absorbed anti-(intact virus, type O_1), an anti-(virus subunit, type O_1), an anti-(isolated virus protein VP1, type O_1), and, as a control, an anti-(intact virus, type C_3). The two anti-intact virus sera tested, scans 1 and 2, show the extremes in the reactivity patterns found. Large quantitative differences in the individual animal responses to an identical antigen preparation have been reported before, but these scans highlight the variability possible in the antibody composition between sera. Examination of scans 1, 2, and 3 shows that antibodies reactive with hexapeptide numbers 146 and 147 are present in anti-intact particle sera (scans 1 and 2) but completely absent after absorption of the sera with purified virus (scan 3). Presumably, scan 3 registers those antibodies raised against epitopes expressed in denatured virions that are not present on the surface of the intact virion. Activities to hexapeptides 146 and 147 were not observed in the anti-subunit serum (scan 4) and were only weakly present in the anti-VP1 serum (scan 5). That some activity was found in the anti-VP1 serum

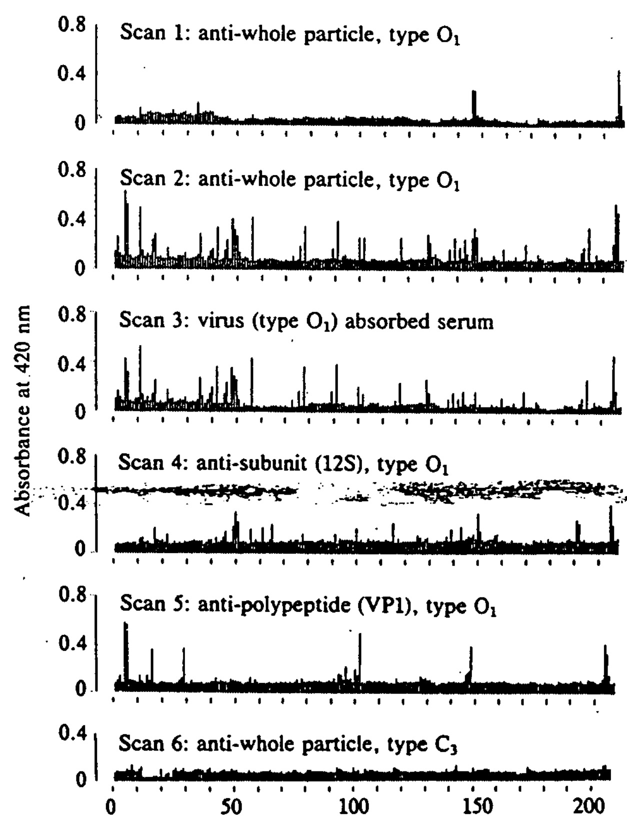

FIG. 2. Antigenic profiles (scans). Results are shown as vertical lines proportional to the extinction obtained in the antibody-binding ELISA test, plotted above the number giving the location within the VP1 sequence of the NH_2-terminal amino acid of each peptide. Antisera used to produce the scans shown were as follows: 1 and 2, two different anti-whole virus particle, type O_1; 3, anti-whole virus particle (as used in 2) after absorption with purified intact virus; 4, anti-virus subunit, type O_1; 5, anti-VP1, type O_1; 6, anti-whole virus particle, type C_3. It should be noted that, because the sequence of VP1 contains 20 alanine residues, 20 of the peptides synthesized match for seven amino acids. However, the frequency of reactive peptides from this group was not significantly different from the overall frequency (0.2 compared with 0.16) and therefore not considered further.

possibly accounts for the immunizing capacity, albeit weak, of the isolated protein (20). It should be noted however that another anti-VP1 serum tested, while retaining a strong activity at position 148, showed no activity at positions 146 and 147. Comparison of scan 3 with scan 2 (absorbed compared with nonabsorbed) shows that, in addition to the loss of activity to peptides 146 and 147, some reduction in activity to peptides 5, 6, and 206 also occurred. Of these, activity to 5 and 6 was not found in all the anti-intact virus sera tested, but activity to 206 was invariably present. From this we conclude that of the peptides found to be reactive, the pair at 146 (G-D-L-Q-V-L) and 147 (D-L-Q-V-L-A) [in this paper, amino acids are identified by the single-letter code (21)] constitute or are part of the principal immunogenic epitope, with the element at 206 (V-A-P-V-K-P) contributing to a lesser epitope. This is consistent with the observations of others (5, 22). Scan 6 shows the absence of reactivity in an antiserum produced against a different serotype of the virus.

Extending the Resolution of the Epitope at Peptides 146/147 to a Single Amino Acid. From the preceding data, we were unable to distinguish between two possibilities: (*i*) the epitope is contained in the five amino acids common to peptides 146 and 147—i.e., D-L-Q-V-L—or (*ii*) the epitope is represented by the "sum" of the two hexapeptides—i.e., G-D-L-Q-V-L-A. To extend the resolution, all 120 possible hexapeptides differing from peptide 146 (G-D-L-Q-V-L) by only a single amino acid were synthesized. Each of the other 19 common amino acids was substituted in each of the six amino acid positions within the peptide. Positions at which all or at least the majority of substitutions result in a loss of antibody-binding activity indicate those residues that are important for the specificity and binding to antibody. The ELISA activity obtained for each of the 120 peptides when serum 48 (anti-intact virus particle) was used in the test are shown in Fig. 3. The relative activities (with respect to the parent sequence) determined for each peptide for two different anti-intact virus sera, nos. 31 and 48, are given in Table 1. To determine the contribution of the alanine residue (carboxyl terminus of peptide 147) toward reactivity and/or specificity, a further 20 peptides were synthesized. Each of these peptides consisted of the complete sequence of 146 (G-D-L-Q-V-L) with one of the 19 possible amino acids added to the carboxyl terminus and synthesized as described before. When serum 31 was used in the test, activity was retained for seven of the amino acids. Relative values expressed in the same way as given in Table 1 were as follows: A (parent amino acid), 99; D, 55; E, 36; G, 45; N, 95; Q, 98; S, 44. With

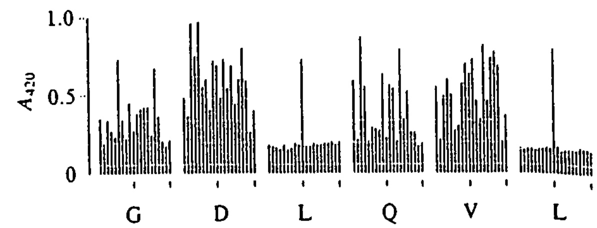

FIG. 3. Antibody-binding activity. The result for each peptide is shown as a vertical line proportional to the ELISA extinction obtained. Every group of 20 lines corresponds to the complete replacement set for one of the six amino acid positions in the hexapeptide G-D-L-Q-V-L. Within each group of 20 lines, the left-hand line corresponds to the substitution of the original residue by alanine (A), and the successive lines are then in alphabetic order according to the single letter code for the amino acids.

serum 48, activity was retained for four amino acids: A (parent amino acid), 94; G, 30; S, 47; T, 39.

DISCUSSION

Interpretation of Data. In choosing to adopt the procedure for peptide synthesis as described, we made several assumptions.

1. To detect antibodies, the quantity of peptide of a defined sequence need only be in the pmol range (5). Assuming a worst-case overall yield of 1% for an eight-step synthesis (two linking and six sequence amino acids), an initial level of 1 nmol of reactive group per support would satisfy the above condition.

2. High purity for the peptide used in the detection of antibodies is not a necessary condition. The majority of serological tests rely on the specificity of antibodies to detect a given antigen in the presence of large amounts of irrelevant protein.

3. Except for cases in which either all or none of the peptides react, a large number of the peptides would effectively act as negative controls in the test. With adjacent peptides sharing a common sequence of five amino acids, the observation of peaks above a generally uniform background level would indicate a valid test.

4. Many of the antibodies elicited by immunization with an intact virus result from presentation of epitopes in fully or partially denatured form. Such antibodies may bind to synthetic peptides *in vitro* but not to the virion itself. They are therefore assumed to be less relevant to virus neutralization

Table 1. Relative antibody-binding activities of peptides derived from the parent sequence G-D-L-Q-V-L

Serum	Parent residue	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
		Activity when substituted with amino acid																			
31	G	29					**90**	14	27	12		32	34	41	29		50				
	D	22	21	**143**	95	110	28	65		12	65	15	58	10	69		38	62			
	L										**79**										
	Q			64	14								13		**80**						
	V	62		33	52				26		29	59			45		49	43	**89**		
	L										**119**										
48	G	11					**88**	10		32		18	24	25	26		77	14			
	D	37	12	**136**	92	137	52	62	21	87	81	37	89	49	80	29	63	104	60		21
	L										**88**										
	Q	60		117	52					68		53	49		**102**	10	45				
	V	52		40	63	42			56	82	68	88	34		106	33	91	98	81		14
	L										**105**										

Antibody-binding activities are shown for all peptides that gave an extinction significantly above background. Values for each peptide are expressed as a percentage of the mean activity of the six parent sequences synthesized as a part of each replacement set. Values given boldface type correspond to those obtained for the parent sequence. No activity was detected when the antiserum used was prepared against the heterologous FMDV type.

than are antibodies that bind to virions as well as to peptides.

The extinction obtained in an ELISA for a given peptide depends on the concentration of the antibody population with the correct specificity for reaction. It is essentially independent of the peptide density expressed as reacting groups per mm^2 of support (unpublished data). The difference in the extinction obtained with peptides synthesized with densities varying over two orders of magnitude is similar to the 10–30% variation observed between replicate synthesis (unpublished data). The extinction may also be expected to depend greatly on the affinity between peptide and reacting antibody, but this remains to be verified, although the overnight reaction would tend to minimize differences. Antigenic profiles of the FMDV VP1 (Fig. 2) were interpreted to define an antigenic peptide as one giving an ELISA extinction significantly above the background level of the test. On the other hand, in the testing of replacement nets (Fig. 3), the concentration of the reactive antibody population is constant and effectively of one specificity. Therefore, the extinctions observed are interpreted to reflect the mean affinity of the reacting antibody population for the peptide.

Immunogenicity of FMDV Virus Protein (VP1). Scan 5 of Fig. 2 identifies six immunogenic regions defined in terms of epitopes on the isolated protein eliciting antibodies capable of binding to the corresponding synthetic peptide. Scan 4 shows that, for the same protein as a part of the virus subunit, additional regions (principally 50–70 and 191–197) are immunogenic. Scan 2 shows that, during the course of the immune response to whole virus, most of the protein can be immunogenic. In contrast, scan 1 shows a response to only a very limited number of epitopes. What has become clear from these and other results (unpublished) is that different animals do not necessarily respond to all of the epitopes on a given antigen. In addition, the immunogenic response of an individual animal will be complicated if the antigen is readily broken down as is known to happen to FMDV (23, 24). The animal is exposed not only to the intact virus but also to subunits and possibly even to the isolated viral proteins. Each of these different states could present different epitopes to the immune system. Epitopes can be identified with a particular state of the antigen by testing the peptides with antisera specific to that state.

An Immunogenic Epitope at High Resolution. Antibodies raised against a particular immunogenic epitope will have a combining site (paratope) complementary to the structure of that epitope. An antibody population directed to the same epitope (allowing for variation in the expression of antibodies by the immune reponse) will have common features in the combining sites essential for binding to that epitope. A peptide that, in one of its many conformations in thermal equilibrium *in vitro*, has a structure sufficiently similar to the form of the epitope against which antibody was raised *in vivo* will bind to the antibody. Modification of a reacting peptide by amino acid substitution will define the limits for interaction with antibody. By so "mapping" the antibody-combining site, it is possible to infer properties of the antigen to which this antibody population is complementary. Using polyclonal antisera, it was not expected that a rigorous requirement for particular amino acids in particular positions would be observed. It is clear that, whatever the diversity of the antibodies involved in the interaction, the requirement for a given amino acid in certain positions is absolute for most or all of the antibodies present. It is also clear that the specificity range found for the two different antisera is remarkably similar, differing mainly in the hierarchy of preference for amino acids at the nonessential position. As judged from the limitation to replacements at some position within the sequence G-D-L-Q-V-L-A, the whole-virus epitope may be considered to be X-X-**L**-Q-X-**L**-A, where X is nonessential, letters in boldface type indicate an absolute requirement, and letters in lightface type indicate a contributing amino acid.

These findings suggest a different interpretation of the characteristics of epitopes. The antigenic specificity of the epitope represented by amino acids 146–152 within the VP1 protein of FMDV is largely dependent on the leucine residues at positions 148 and 151. These are hydrophobic residues and would not normally be expected to protrude from the protein surface. This suggests the possibility that the immune system responds to a local protein conformation that is different from that expected to represent the global energy minimum. The energy for antigen–antibody binding may be derived from the positive entropy term associated with the transfer of hydrophobic residues from a hydrophilic (aqueous) environment to within the antibody-combining site.

Scope of the Described Approach to Epitope Mapping. Although our results have been presented for a single protein only, the agreement with results of others in locating a viral epitope within the region encompassing amino acids 141–160 of VP1 is excellent (5, 22). The further resolution obtained by Rowlands *et al.* (25) from the comparison of the sequences of the VP1s of three antigenic variants of a single virus type (A_{12}) showed that amino acid substitution at positions 148 and/or 153 would affect the ability to react with specific antibody. This result is in good agreement with our results for subtype O_1, where positions 148 and 151 were critical to the immunogenicity of the epitope. We expect that the systematic approach as outlined, when applied to a broader spectrum of proteins, will contribute greatly to our understanding of the nature of epitopes and their interaction with the immune system.

We thank Mr. Jan Briaire for his enthusiastic and skilled technical assistance with the synthesis of the peptides, Mr. Jan Meyer for assistance with the ELISA, and Dr. Dick Voskamp of the Technical University, Delft, for valuable advice on aspects of the peptide chemistry. This work is the result of a collaborative project between the Commonwealth Serum laboratories and the Central Veterinary Institute and was conducted at the latter institute.

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Selection of Immunogenic Peptides for Antisera Production

In order to produce antisera reactive with proteins, a peptide is selected from a translated cDNA or protein sequence and is then synthesized, purified (at least partially), and conjugated to a carrier protein. Strategies for selecting immunogenic peptides and carrier proteins as well as various means of coupling peptides to carriers are described. The actual coupling protocols, a typical immunization protocol, and a procedure for preparing a peptide affinity column for antibody purification are included in *UNIT 9.4*. A flowchart for the preparation of antipeptide antibodies (antibodies reactive with a synthetic peptide) is shown in Figure 9.3.1.

SELECTION OF AN IMMUNOGENIC PEPTIDE

To prepare antibodies against synthetic peptides, a peptide must first be selected. This is the most critical step in obtaining an antibody that reacts with the native antigen. In practice, a 10- to 15-residue peptide sequence inferred from a cDNA sequence or from an N-terminal amino acid sequence is selected. If possible, sequences should be avoided that are likely to be identical or highly homologous to those in the animal to be immunized (usually rabbits). After synthesis and purification, the peptide is cross-linked to a carrier protein such as keyhole limpet hemocyanin (KLH).

If a peptide sequence to be utilized for antisera production is not from the terminal regions of the protein, selection is based on predicting antigenic sites. It is presumed that the sites accessible to reactivity with antipeptide antibodies are exposed on the surface of the protein; these sites are likely to be more common in flexible regions of the protein (Westhof et al., 1984), and are more likely to be found on reverse turns or loop structures (Dyson et al., 1985). Computer algorithms

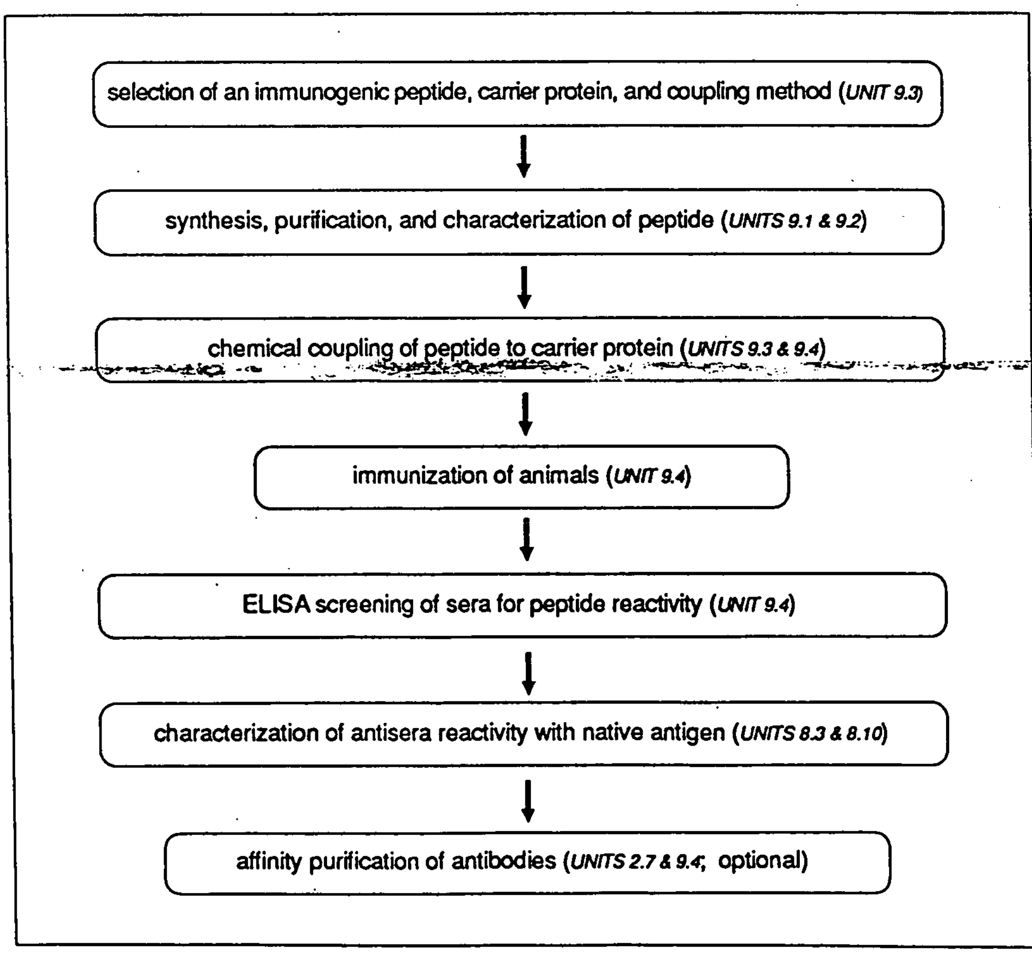

Figure 9.3.1 Flow chart for preparation and analysis of antipeptide antibodies.

estimate hydrophilicity (Kyte and Doolittle, 1982; Hopp and Woods, 1983), flexibility (Karplus and Schultz, 1985), and secondary structure of the protein along its polypeptide chain; they can help identify surfaces on the protein that have appropriate characteristics for antigenic sites. Antisera produced against peptides derived from such regions are more likely to react with the native protein than peptides derived from regions without these characteristics.

Selection of a C-Terminal Peptide

Because the C termini of proteins are often more mobile than the rest of the molecule and are frequently exposed on the protein surface, this region is usually accessible to antibody-combining sites. This is particularly true for detergent-solubilized transmembrane proteins with C-terminal cytoplasmic tails—i.e., MHC class I and class II molecules, and T cell receptor molecules. This type of peptide can be coupled to the carrier in a straightforward manner using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) via a Cys residue that has been added to the N terminus of the selected peptide. By coupling the peptide via its N-terminal end to the carrier protein, the peptide will be exposed in a fashion similar to that found in the native antigen. Obviously, if any other Cys residues are present, they will also couple; therefore, an alternate coupling procedure may be more appropriate (see coupling methods).

Unfortunately there will be instances when the C-terminal sequence will not be the preferable immunogen—e.g., if its sequence is part of a transmembrane region and is thus too hydrophobic. This can be determined by examining a hydropathicity plot of the sequence (Kyte and Doolittle, 1982).

Selection of an N-Terminal Peptide

As with the C terminus, the N terminus is often exposed in the native protein; therefore, peptides selected from this region are often useful for making antibodies reactive with the intact protein. In this case, if MBS coupling is used, the Cys residue should be present on the C terminus. By coupling the peptide via the C-terminal end to the carrier protein, it will be oriented as it is in the native protein. Unfortunately, the N terminus of a protein may have a post-translational modification, such as acetylation or fatty acid acylation. Moreover, if the protein sequences are derived from cDNA sequences, the leader sequence must not be confused with the authentic N terminus. It is usually possible to locate potential leader sequences using an algorithm derived by von Heijne (1986). If it is known that the authentic N terminus is acetylated, the peptide can then be acetylated during synthesis to reproduce the structure in the native protein.

Selection of an Internal Peptide Sequence

Selection of a peptide from an internal part of the protein sequence can be aided by the use of algorithms to predict those regions most likely to be exposed on the surface of the protein. The only information needed is the primary amino acid sequence. Two of the methods discussed below are based on the fact that antibody-reactive sites are usually located in externally exposed, hydrophilic regions of proteins. A third method relies on secondary structure predictions.

Using the first algorithmic method, a hydrophilicity value is assigned for each overlapping six-amino-acid segment of the protein sequence based on the average of the hydrophilicity values (Table 9.3.1) of the amino acids in that segment. The highest point of average local hydrophilicity is usually located in or near an antigenic determinant. A computer program written in Basic is available for analysis (Hopp and Woods, 1983).

An alternative algorithm evaluates the hydrophobic and hydrophilic tendencies of a polypeptide chain based on water vapor free-energy transfers and the interior versus exterior distributions of amino acid side chains. Values for each amino acid are listed in the second column of Table 9.3.1. A computer program for calculating this hydropathicity profile has been written by Kyte and Doolittle (1982). This profile is useful for determining exterior and interior regions of a native protein, as well as for locating signal sequences and transmembrane sequences.

A third algorithm is an empirical method that relies on a library of known structures to determine the frequency with which each amino acid occurs in the various conformational states (i.e., α-helix, β-sheet, β-turn, or all other structural forms; Chou and Fasman, 1974). Using these frequencies, predictions can be made about secondary structure for a given sequence. For making antipeptide sera, regions that are predicted to form turns or loops, or extended sequences (20 to 25 residues) that have a very high probability for formation of an α-helix, are useful. A computer program for performing this analysis can be found in

Corrigan and Huang (1982).

Although no data exists to prove this point, it would seem wise to avoid choosing peptides containing predicted polysaccharide attachment sites, most notably the sequence Asn-X-Ser or Asn-X-Thr, which predict the presence of an Asn-linked polysaccharide. It is likely that the presence of polysaccharide moieties at such sites in the native protein would interfere with antibody accessibility.

The above-mentioned computer programs, plus programs to predict flexibility, location of transmembrane regions, Asn-linked glycosylation sites, and sites of signal sequence cleavage, are all contained in a package called PC Gene produced by IntelliGenetics (*APPENDIX 5*).

Selection of the Length of the Peptide

Generally, peptides with a length of 10 to 15 residues are used to make antipeptide sera that react with the native protein. Peptides with as few as 6 or as many as 35 amino acids have worked successfully; however, both extremes have disadvantages. Small peptides are more soluble and can produce very specific antisera, but the antibodies elicited by them are not as likely to react with the parent protein. Large peptides tend to be less soluble, more difficult to prepare synthetically, and are more likely to assume structures unrelated to the native protein. Part of the decision about peptide size will be determined by the individual peptide sequence, as some residues will adversely affect solubility, produce synthesis problems, or interfere with coupling.

Thus, in summary, a reasonable order of suggestions for choosing peptide sequences for making antipeptide sera would be:

1. If possible, use more than one peptide.
2. Use the C-terminal sequence (7 to 15 residues) if it is hydrophilic and if a suitable coupling group is available or can be added.
3. Use the N-terminal sequence (7 to 15 residues) if it is hydrophilic and if a suitable coupling group is available or can be added.
4. Use internal hydrophilic regions, perhaps using longer peptides (15 to 20 residues).

Modification of the Chosen Peptide

Other features of the peptide must be considered in order for it to mimic the native antigen as closely as possible. If the desired peptide sequence comes from an internal portion of the native protein, then the free N-terminal amino and C-terminal carboxyl groups (which are normally peptide-bonded to adjacent amino acid residues within the native protein) can be modified to more closely

Table 9.3.1 Hydrophobic/Hydrophilic Index of Amino Acids

Amino acid	Hydrophilicity value[a]	Hydropathy index[b]
Arginine (R)	3.0	–4.5
Aspartic acid (D)	3.0	–3.5
Glutamic acid(E)	3.0	–3.5
Lysine (K)	3.0	–3.9
Serine(S)	0.3	–0.8
Asparagine (N)	0.2	–3.5
Glutamine (Q)	0.2	–3.5
Glycine (G)	0.0	–0.4
Proline (P)	0.0	–1.6
Threonine (T)	–0.4	–0.7
Alanine (A)	–0.5	1.8
Histidine (H)	–0.5	–3.2
Cysteine (C)	–1.0	2.5
Methionine (M)	–1.3	1.9
Valine (V)	–1.5	4.2
Isoleucine (I)	–1.8	4.5
Leucine (L)	–1.8	3.8
Tyrosine (Y)	–2.3	–1.3
Phenylalanine (F)	–2.5	2.8
Tryptophan (W)	–3.4	–0.9

[a]Hopp and Woods (1981).
[b]Kyte and Doolittle (1982).

Table 9.3.2 Principal Carriers Used for Coupling Peptides[a]

Carrier[b]	M_r (kDa)	Number of groups/molecule			
		ε-NH_2	-SH	Phenol	Imidazole
BSA	67	59[c]	1	19	17
Ovalbumin	43	20	4	10	7
Myoglobin	17	19	0	3	12
Tetanus toxoid	150	106	10	81	14
KLH	>2000	6.9[d]	1.7[d]	7.0[d]	8.7[d]

[a]Adapted from Van Regenmortel et al. (1988).
[b]Abbreviations; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.
[c]Only 30 to 35 of the 59 Lys residues of BSA are accessible.
[d]For KLH, the amino acid groups are expressed in grams of amino acid containing this functional group per 100 g.

mimic their native structure. The N-terminal amino group can be modified by acetylation of the peptide α-amino group during synthesis, and the C-terminal carboxyl group can be modified with a C-terminal amide during peptide synthesis. It is not certain that acetylation of the N terminus or formation of the C-terminal amide for peptides derived from internal sequences will really improve the chances of producing antisera reactive with the native protein. However, it has been demonstrated that these modifications will stabilize an α-helical conformation and may increase the solubility of the peptide.

SELECTION OF A CARRIER PROTEIN

A carrier protein should be a good immunogen and have a sufficient number of amino acid residues with reactive side-chains (see Table 9.3.2) for coupling to the synthetic peptide. KLH is commonly used because of its proven efficacy. However, because of its large size, KLH is more likely to precipitate during cross-linking, making the complex difficult to handle. Other proteins that have been used as carrier molecules include thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. BSA has the disadvantage that anti-carrier protein antibodies present in the anti-peptide serum will be a problem if the antiserum is used in the presence of fetal calf serum. Table 9.3.2 lists the most common protein carriers and their relevant properties.

SELECTION OF A COUPLING METHOD

In addition to the choice of peptide, a method for coupling the peptide to a protein carrier must also be selected. Most coupling methods rely on the presence of free amino (Lys), sulfhydyl (Cys), phenolic (Tyr), or carboxylic (Asp or Glu) groups. The chosen coupling method should link the peptide to the carrier via either the C- or N-terminal residue. Peptides corresponding to the amino terminus of proteins should be coupled through their carboxyl-terminal amino acid residue, whereas peptides corresponding to the carboxyl terminus of proteins should be coupled through their amino-terminal amino acid residue.

One coupling procedure that has proved to be particularly effective employs MBS as the coupling reagent. This procedure requires a free sulfhydryl group on the synthetic peptide and free amino groups on the carrier protein. Therefore, in order to use this method, it is usually necessary to add a Cys residue (during peptide synthesis) to the C or N terminus of the peptide. This will provide the sulfhydryl group for coupling to the carrier protein. Coupling a peptide derived from an N-terminal sequence to a carrier is accomplished with MBS via a Cys residue added to the C terminus of the peptide. This cross-links the peptide derived from the N-terminal sequence to the carrier molecule so that the N terminus is exposed as it would be in the native antigen. For a peptide derived from a C-terminal sequence, the Cys is placed on the N-terminus of the peptide for the same rationale. If an internal Cys residue is present in the chosen peptide, it can be used for MBS coupling, especially if the cysteine is part of a disulfide linkage in the native protein.

If coupling with MBS is not desirable for some reason (such as the presence of non-terminal Cys residues that are not disulfide-linked in the native protein), coupling can then be accomplished through a C- or N-terminal Tyr using *bis*-diazotized benzidine (BDB), through the C or N terminus with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI), or

Table 9.3.3 Principal Reagents Used for Peptide Protein Conjugation[a]

Coupling agent	Modified amino acid	
	Primary reaction	Secondary reaction
Glutaraldehyde	ε-NH_2Lys, α-NH_2, SH-Cys	Tyr, His
Bis-imido esters	α-NH_2, ε-NH_2Lys	Negligible
BDB	Tyr, SH-Cys, His, ε-NH_2Lys	Trp, Arg
Carbodiimides (EDCI)	α-NH_2, ε-NH_2Lys, α-COOH, Glu, Asp	Tyr, Cys
MBS	Cys-SH	Not observed

[a]Adapted from Van Regenmortel et al. (1988). Abbreviations: BDB, *bis*-diazotized benzidine; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide;MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide.

through the N-terminal α-amino group with glutaraldehyde. BDB coupling is not advisable if there are internal Tyr residues in the peptide. EDCI coupling is not advisable when internal Glu, Asp, or Lys residues are present. Glutaraldehyde coupling may not be appropriate if there are internal Lys residues in the peptide. Table 9.3.3 lists the principal reagents used for peptide-protein conjugation and the functional groups involved.

For example, if the carboxy-terminal sequence of a protein is

1 2 3 4 5 6 7 8 9 10
S Y G R N Q A E C Q—COOH

then coupling via MBS by adding a Cys residue to the N terminus may not be appropriate because of the Cys at position 9 (see Table 9.3.1 for single-letter codes). In this case, it may be preferable to couple the peptide via the N terminus using glutaraldehyde. However, if the Cys at position 9 is known to be part of a disulfide loop in the native protein, it may be better to couple with MBS through the naturally occurring Cys at position 9. Protocols for the coupling methods discussed here are presented in UNIT 9.4.

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KEY REFERENCE

Van Regenmortel et al., 1988. See above.

Presents detailed discussion of selection of peptides for preparing antipeptide sera reactive with native proteins.

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